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# Developing the role of PCR for detection and characterisation of helicobacters in human infection

by

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Thesis submitted for the Degree of Doctor of Philosophy

July 2003


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Submission date: 11 August 2003
Award date: 13 February 2004

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*I dedicate this thesis to my parents.*

**Abstract**

This study aimed to explore and develop the role of PCR in the investigation of human *Helicobacter* infections. Overall performance of PCR assays (both conventional and real-time) was influenced by certain key factors, notably specimen transport conditions, DNA extraction methods, primers, and inherent target sequence variation. PCR-based detection and characterisation of non-viable *H. pylori* in gastric biopsies demonstrated the benefit of this approach as an alternative to culture, while the design and application of the first PCR test to detect both *H. pylori* and '*H. heilmannii*'-like organisms (HHLOs) showed a higher rate of HHLO infection (2.3 %) than had been reported previously. The potential of PCR for further strain characterisation was demonstrated by determining clarithromycin susceptibility direct from gastric biopsy by a real-time PCR (LightCycler) assay for mutation detection. Development of an analogous test for metronidazole appears less feasible, as examination of a unique strain set suggested that, contrary to earlier reports, genetic mutations in *rdxA* and *frxA* may not be the principal resistance mechanism. Additionally, development of a novel multiplex PCR assay to determine *vacA* genotypes direct from gastric biopsies provided a useful tool to facilitate surveillance and confirmed that the s1m1 genotype is a common feature of peptic ulcer disease-associated isolates. Application of three novel LightCycler PCR assays to detect *cagA* tyrosine phosphorylation motifs (TPMs) demonstrated that TPM A was common in South East England. No significant associations were observed between disease status and *cagA* TPM. Extension of the PCR-based approach for non-invasive detection of *H. pylori* from stool specimens showed that it was less sensitive and less specific than stool antigen testing. Nevertheless, development of real-time nested PCRs that performed identically to conventional assays demonstrated the potential for next-day diagnosis from stools and offered the possibility of future screening of larger populations. Application of PCR to investigate extra-gastric chronic inflammatory conditions provided the first evidence of *H. pylori* DNA in the human bladder, although its significance as a causal factor in human disease remains unclear. The study provides new evidence to support wider use of conventional and real-time PCR-based assays as powerful tools for future investigation of gastric and extra-gastric *Helicobacter* infections.

**Acknowledgements**

I wish to thank my supervisor, Dr Robert Owen, for giving me the opportunity to register for this degree while employed in his unit and for his encouragement and guidance throughout this project. Thank-you also to my external supervisor, Professor Jeremy Hardie for his enthusiasm and helpful comments. I am extremely grateful to Dr Janet Gibson for her invaluable advice and expertise in the early phases of this project, and to Dr Andy Lawson for his encouragement and advice while I was writing this thesis. I would also like to thank all members of the Helicobacter Reference Unit (past and present!), particularly Miss Sally Sharp, Miss Nic Elviss and Miss Claire Watson for their collaboration and assistance. Thank you also to all external collaborators, for their clinical expertise and hard work collecting the clinical samples. This project would not have been possible without their input and co-operation. I wish also to thank other colleagues in the Laboratory of Enteric Pathogens, particularly Dr Henry Smith for his continued interest in my progress and Dr Henrik Chart for his enthusiastic organisation of the Postgraduate Group and for his co-ordination of registration administration. Finally, thank you to my family and friends for tolerating my absence from social events over the last year!

This research was funded by a PHLS research and development grant.

## Declaration

No part of this thesis has been submitted in support of an application for any higher degree or qualification of any other university or institute of learning. The work of this thesis was performed by myself unless otherwise stated in the text.

Stephanie Chisholm

July 2003

## Publications, presentations and awards arising from this thesis

### Publications

1. **Chisholm, S. A.**, Owen, R. J., Teare, E. L. and Saverymuttu, S. (2001) PCR-based diagnosis of *Helicobacter pylori* infection and real-time determination of clarithromycin resistance directly from human gastric biopsy samples. *Journal of Clinical Microbiology* **39** (4), 1217-1220.

2. **Chisholm, S.A.** and Owen, R.J. (2002) Determination of *Helicobacter pylori vacA* allelic types by single-step multiplex PCR. . *Letters in Applied Microbiology* **35**, 42-46.

3. **Chisholm, S.A.** and Owen, R.J. (2003) Development and application of a novel screening PCR assay for direct detection of '*Helicobacter heilmannii*'-like organisms in human gastric biopsies in Southeast England. *Diagnostic Microbiology and Infectious Diseases* **46**, 1-7.

4. **Chisholm, S.A.** and Owen, R.J. (2003) Mutations in *Helicobacter pylori rdxA* gene sequences may not contribute to metronidazole resistance. *Journal of Antimicrobial Chemotherapy* **51**, 995-999.

5. Owen, R.J., Sharp, S.I., **Chisholm, S.A.** and Rijpkema, S. (2003) Identification of *Helicobacter pylori cagA* tyrosine phosphorylation motifs in DNA from isolates infecting peptic ulcer patients in England by novel RFLP and real-time fluorescence PCR assays. *Journal of Clinical Microbiology.* **41**, 3112-3118.

6. Bell, S.J., **Chisholm, S.A.**, Owen, R.J., Borriello, S.P. and Kamm, M.A. (2003) Evaluation of *Helicobacter* species in inflammatory bowel disease. *Alimentary Pharmacology & Therapeutics.* **18**, 481-486.

7. **Chisholm, S.A.** and Owen, R.J. (2003) Frame-shift mutations in *frxA* occur frequently and do not provide a reliable marker for metronidazole resistance in UK isolates of *Helicobacter pylori. Journal of Medical Microbiology.* In press.

**Chisholm, S.A.**, Watson, C.L., Teare, E.L., Saverymuttu, S and Owen, R.J. A comparison of three commercial stool antigen detection tests for non-invasive diagnosis of *Helicobacter pylori* infection in South East England. Submitted (2003) to *Journal of Medical Microbiology.*

**Oral Presentations**

1. PHLS 24th Annual Scientific Conference, Warwick, September 1999.
   - "Novel method for rapid determination of clarithromycin sensitivity in *Helicobacter pylori*"
2. Royal Society of Medicine, Section of Pathology, Presidents Prize Evening, London, March 2000.
   - "Novel PCR-based assays for *Helicobacter pylori* detection and determination of clarithromycin susceptibility direct from gastric biopsy"
3. Society for Applied Microbiology Symposium on *Campylobacter*, *Helicobacter* and *Arcobacter*, Glasgow, July 2000.
   - **Chisholm, S. A.**, Gibson, J. R., Teare, E. L. and R. J. Owen. "Novel PCR-based assay for the detection of *Helicobacter pylori* and rapid real-time PCR determination of clarithromycin susceptibility direct from biopsy" *J Appl Microbiol* **89** Suppl. O6.
4. PHLS 25th Annual Scientific Conference, Warwick, September 2000
   - **Chisholm, S. A.**, Gibson, J. R. and Owen, R. J. "Novel PCR-based assays for *Helicobacter pylori* detection and determination of clarithromycin susceptibility direct from gastric biopsy"
5. XIIIth International Workshop on Gastroduodenal Pathology and *Helicobacter pylori*, Rome, October 2000.
   - **Chisholm, S. A.**, Owen, R. J. and Teare, E. L.. "Novel PCR-based assays for *Helicobacter pylori* detection and determination of clarithromycin susceptibility direct from gastric biopsy" *Gut* **47** Suppl 1 A123.
6. 11th International Workshop on Campylobacter, Helicobacter and Related Organisms Freiburg September 2001.
   - **Chisholm, S. A.** and Owen, R. J. "Development and evaluation of a novel multiplex PCR assay for diagnosis of *Helicobacter pylori* and *Helicobacter heilmannii* infections of the gastric mucosa" *Int J of Med Microbiol* **291** Suppl 31 47.
   - **Chisholm, S. A.** and Owen, R. J.. "Metronidazole resistance in *Helicobacter pylori*: are mutations in *rdxA* of any significance?" *Int J of Med Microbiol* **291** Suppl 31 15.
   - Owen R.J., Sharp, S.I., Rijpkema, S. and **Chisholm, S.A.** "PCR-based assays for identification of *Helicobacter pylori cagA* tyrosine phosphorylation motifs (TPM) and frequencies in isolates from peptic ulcers and other gastric diseases" *Int J of Med Microbiol* **291** Suppl 31 30.
7. XIVth International Workshop on Gastroduodenal Pathology and *Helicobacter pylori* Strasbourg September 2001.
   - **Chisholm, S. A.** and Owen, R. J.. "Development and evaluation of a novel multiplex PCR assay for diagnosis of *Helicobacter pylori* and *Helicobacter heilmannii* infections of the gastric mucosa" *Gut* **49** Suppl II A98.
   - Owen R.J., Sharp S.I., Rijpkema, S. and **Chisholm, S.A.** "PCR-based assays for identification of *Helicobacter pylori cagA* tyrosine phosphorylation motifs (TPM)

and frequencies in isolates from peptic ulcers and other gastric diseases" *Gut* **49** Suppl II A15.

8. 4th European Congress of Chemotherapy and Infection, Paris May 2002.
   - **Chisholm, S. A.** and Owen, R. J. "Contribution of *rdxA* and *frxA* mutations to metronidazole resistance in *Helicobacter pylori*: an examination of multiple-isolate patient sets" *Int J of Antimicrobial Agents* **19** Suppl 1 S2.

**Poster presentations**

1. XIIIth International Workshop on Gastroduodenal Pathology and *Helicobacter pylori*, Rome, October 2000.
   - **Chisholm, S. A.**, Owen, R. J., Teare, E. L., Anjum, F.H. and Lewi H. 2000. "PCR-based detection of *Helicobacter spp.* in bladder biopsies: a novel risk factor for patients with chronic interstitial cystitis?" *Gut* **47** Suppl 1 A90.
2. 11th International Workshop on Campylobacter, Helicobacter and Related Organisms Freiburg September 2001.
   - **Chisholm, S. A.** and Owen, R. J. Determination of *vacA* genotype direct from gastric biopsies by a single-step multiplex PCR system" *Int J of Med Microbiol* **291** Suppl 31 47.
3. XIVth International Workshop on Gastroduodenal Pathology and *Helicobacter pylori* Strasbourg September 2001.
   - **Chisholm, S. A.** and Owen, R. J. "Determination of *vacA* genotype direct from gastric biopsies by a single-step multiplex PCR system" *Gut* **49** Suppl II A16.
4. XVth International Workshop on Gastroduodenal Pathology and *Helicobacter pylori*, Athens, September 2002.
   - **Chisholm, S. A.** and Owen, R. J. "Potential involvement of alkylhydroperoxide reductase (AhpC) in metronidazole activity in UK isolates of *Helicobacter pylori* – development of resistance is unlikely to be due to mutational alteration of the *ahpC* gene" *Gut* **51** Suppl II A10.
   - **Chisholm, S.A.** and Owen, R.J. "Frame-shift mutations in *frxA*: A frequent occurrence that may be unrelated to metronidazole resistance in UK isolates of *Helicobacter pylori*" *Gut* **51** Suppl II A11.
   - **Chisholm, S. A.**, Watson, C. L., Teare, E. L., Saverymuttu, S.and Owen, R. J. "Non-invasive diagnosis of *Helicobacter pylori* infection in Southeast England: A comparative evaluation of two commercial kits, HpSA and HpStAR, for stool antigen detection" *Gut* **51** Suppl II A107.

**Awards**

- Royal Society of Medicine President's Prize, March 2000.
- BSAC Young Investigators award to attend 4th European Congress of Chemotherapy and Infection, Paris May 2002.
- CPHL Travel Fellowship 2003.

**Glossary of abbreviations**

| | |
|---|---|
| A | Adenine |
| AFLP | Amplified Fragment Length Polymorphism |
| Ala | Alanine |
| AMO | Amoxicillin |
| Arg | Arginine |
| BB | Brucella Broth |
| BIS | Bismuth Salts |
| BSA | Bovine Serum Albumin |
| BLASTn | Basic Alignment Sequence Tool nucleotide |
| C | Cytosine |
| CBA | Columbia Blood Agar |
| CD | Crohn's Disease |
| cDNA | Copy Deoxyribonucleic Acid |
| CFU | Colony Forming Units |
| CHD | Chronic Heart Disease |
| CLA | Clarithromycin |
| CLA-S | Clarithromycin Sensitive |
| CLA-R | Clarithromycin Resistant |
| CLO | *Campylobacter*-Like Organism |
| CPC | Chronic Infant Death Syndrome |
| CPHL | Central Public Health Laboratory |
| CT | Crossover Threshold |
| CTAB | Cetyltrimethyl Ammonium Bromide |
| dNTPs | Deoxynucleotide Trisphosphates |
| ddNTPs | Dideoxynucleotide Trisphosphates |
| DNA | Deoxyribonucleic acid |
| DU | Duodenal Ulcer |
| EDTA | Ethylene Diamine Tetraacetic Acid |
| EHPSG | European *Helicobacter pylori* Study Group |
| ELISA | Enzyme Linked Immunosorbent Assay |
| FISH | Fluorescent In Situ Hybridisation |
| FRET | Fluorescence Energy Resonance Transfer |
| G | Guanine |
| GC | Gastric Carcinoma |
| GERD | Gastro-oesophageal Reflux Disease |
| GU | Gastric Ulcer |
| GuSCN | Guanidium Isothiocyanate |
| HHLO | '*Helicobacter heilmannii*'-Like Organism |
| His | Histidine |
| HpNAP | *H. pylori* Neutrophil Activating Protein |
| HRU | Helicobacter Reference Unit |
| IBD | Inflammatory Bowel Disease |
| IC | Interstitial Cystitis |
| IgA | Immunoglobulin A |

| | |
|---|---|
| IgG | Immunoglobulin G |
| IMS | Immuno Magnetic Separation |
| Leu | Leucine |
| LiPa | Line Probe Assay |
| LPS | Lipopolysaccharide |
| MALT | Mucosal Associated Lymphoid Tissue |
| MIC | Minimum Inhibitory Concentration |
| MTZ | Metronidazole |
| MTZ-S | Metronidazole Sensitive |
| MTZ-R | Metronidazole Resistant |
| MTZCBA | Metronidazole Columbia Blood Agar |
| NADPH | Nicotinamide Adenine Dinucleotide Phosphate (reduced form) |
| NCLD | Non Cholestatic Liver Disease |
| NCTC | National Collection of Type Cultures |
| NIH | National Institute of Health |
| NUD | Non Ulcer Dyspepsia |
| OD | Optical Density |
| ORF | Open Reading Frame |
| PAI | Pathogenicity Island |
| PBC | Primary Biliary Cirrhosis |
| PCR | Polymerase Chain Reaction |
| PHL | Public Health Laboratory |
| POR | Pyruvate Oxidoreductase |
| PPIs | Proton Pump Inhibitors |
| Pro | Proline |
| PSC | Primary Sclerosing Cholangitis |
| PUD | Peptic Ulcer Disease |
| PVP | Polyvinyl Pyrrolidone |
| RE | Restriction Endonuclease |
| RFLP | Restriction Fragment Length Polymorphism |
| rRNA | ribosomal Ribonucleic Acid |
| RT-PCR | Reverse Transcription Polymerase Chain Reaction |
| RUT | Rapid Urease Test |
| SDS | Sodium Dodecylsulphate |
| SIDS | Sudden Infant Death Syndrome |
| T | Thymine |
| Ta | Annealing Temperature |
| TET | Tetracycline |
| Tm | Melting Temperature |
| TPM | Tyrosine Phosphorylation Motif |
| Thr | Threonine |
| UBT | Urea Breath Test |
| UC | Ulcerative Colitis |
| VAIN | Variable Atmospheric Incubator |
| Val | Valine |
| 5-ASA | 5-Aminosalicyclic Acid |

# Chapter 1: INTRODUCTION

## 1.1 History of *Helicobacter pylori*

The first description of gastric spiral organisms in animals can probably be attributed to Bottcher in 1875. At the end of the 19$^{th}$ century, 'Spirochaetes' in the stomachs of dogs and of rodents were reported by Bizzozero and Salomon, respectively. Human gastric spiral organisms were not reported until the early 20$^{th}$ century when, in 1906, Krienitz identified spiral bacteria in the gastric contents of a patient with gastric carcinoma (Kidd and Modlin 1998).

Gastric spiral organisms received little further attention until spiral bacteria observed on the gastric mucosa epithelial surface were proposed to influence polymorphonuclear white blood cell migration (Steer 1975), but these were subsequently misidentified as *Pseudomonas aeruginosa* (Steer and Colin-Jones 1975). Robin Warren, an Australian pathologist, also had observed spiral organisms in histopathological specimens for some years and believed that these may play a role in gastric disease: a controversial view at that time (Kidd and Modlin 1998). In 1983 spiral "*Campylobacter*-like" organisms were cultured for the first time from human gastric biopsies by Warren and his colleague Barry Marshall (Warren and Marshall 1983). The bacteria were present in the majority of patients with chronic active gastritis or ulcers of the stomach or duodenum, and a possible causative role in the development of these diseases was proposed (Marshall and Warren 1984). Initially this organism was named "*Campylobacter pyloridis*" on the basis of guanine-cytosine (G + C) content and cell morphology (Marshall and Warren 1984). A later grammatical correction renamed the organism "*Campylobacter pylori*" (Marshall and Goodwin, 1987). It soon became apparent that *C. pylori* was ultrastructurally atypical

of campylobacters and possessed a distinct fatty acid profile and flagellar apparatus (Goodwin *et al.* 1985). Moreover, partial sequencing and DNA-16S rRNA hybridisation analysis clearly distinguished *C. pylori* from other species of *Campylobacter* (Bukholm *et al.* 1989;Lau *et al.* 1987;Romaniuk *et al.* 1987;Thompson *et al.* 1988). This evidence suggested that *C. pylori* was sufficiently different, both biochemically and genetically, to merit creation of a new genus, *Helicobacter* (Anonymous 1989;Goodwin *et al.* 1989).

## 1.2 The Genus *Helicobacter*

*Helicobacter* and its closest relatives that include *Campylobacter* and *Arcobacter* collectively belong to the class *Epsilobacteria*, that is part of the subdivision *Thiobacteria* of the division *Proteobacteria* (On *et al.* 2002). Since it was first described (Goodwin *et al.* 1989), the genus *Helicobacter* has expanded substantially and at present comprises 22 formally named species (http://www.bacterio.cict.fr), and at least 35 additional novel helicobacters awaiting formal naming (Fox 2002). Helicobacters principally occupy gastric, intestinal and hepatobiliary niches and have been recovered from a wide range of mammals and birds (Table 1.1). A subset of these have been isolated from humans and have in some cases been associated with specific gastric and extra-gastric disease (O'Rourke *et al.* 2001). These other helicobacters are described in sections 1.13 and 1.14.

**Table 1.1** **Gastric and enterohepatic members of the *Helicobacter* genus.**

(Compiled from Fox *et al.* 2002;Fox 2002;Patterson *et al.* 2000;Robertson *et al.* 2001;Solnick and Schauer 2001;Won *et al.* 2002)

| **Gastric *Helicobacter* taxa** | | **Enterohepatic *Helicobacter* taxa** | |
|---|---|---|---|
| *Species* | **Natural host** | *Species* | **Natural host** |
| *H. pylori** | Humans | *H. cinaedi*† | Humans, hamsters |
| *'H.heilmannii'* | Humans, pigs, cats, dogs, monkeys, cheetahs | *H. fennelliae* | Humans, dogs, macaques |
| *'H. rappini'* | Humans, sheep, dogs, mice | *H. pullorum* | Humans, chickens |
| *'H. suncus'* | House musk shrews | *H. canis* | Humans, dogs, cats |
| *H. acinonychis* | Cheetahs | *H. canadensis* | Humans |
| *H. mustelae* | Ferrets | *'H. winghamensis'* | Humans |
| *H. felis* | Cats, dogs, cheetahs | *H. pametensis* | Wild birds, pigs |
| *H. bizzozeronii* | Cats, dogs | *H. bilis* | Mice, dogs, cats |
| *H. salomonis* | Cats, dogs | *'H. marmotae'* | Woodchucks, cats |
| *Candidatus* H. suis | Pigs | *H. hepaticus* | Mice |
| *Candidatus* H. bovis | Cattle | *H. rodentium* | Mice |
| | | *H. typhlonius* | Mice |
| | | *H. ganmani* | Mice |
| | | *H. muridarum* | Mice, rats |
| | | *'H. muricola'* | Korean wild mouse |
| | | *H. trogontum* | Rats |
| | | *H. mesocricetorum* | Hamsters |
| | | *H. cholecystus* | Hamsters |
| | | *H. aurati* | Syrian hamsters |

*This species now includes *H. nemestrinae.*

†This species now includes '*H. mainz*' and '*H. westmeadii*'.

### 1.2.1 Biochemical characteristics of *H. pylori*

The original report by Warren and Marshall described *H. pylori* as a curved Gram negative rod (2.5 μm x 0.5 μm) that possessed up to five sheathed unipolar flagella. The bacterium was also shown to be slow-growing and relatively fastidious, requiring prolonged incubation of 3 - 4 days at 37 °C under microaerophilic conditions (Warren

and Marshall 1983). Further investigation demonstrated that these bacteria were positive for oxidase, catalase and $H_2S$ production but negative for indole and nitrate, and that the DNA G + C content was similar to that of campylobacters (36 % mol) (Marshall and Warren 1984). Although mis-leadingly described in this study as urease-negative, potent urease activity is characteristic of *H. pylori* and this has since become a key biochemical test in its identification, along with the demonstration of catalase and cytochrome oxidase activity.

### 1.2.2 Characteristics of the *H. pylori* genome

Studies throughout the early 1990s demonstrated that the *H. pylori* genome ranged in size from 1.6 to 1.73 M bp and displays much inter-strain diversity in terms of gene order (Ge and Taylor 1999). Comparison of the two *H. pylori* strains for which the entire genome sequence is now described, namely, 26695 (Tomb *et al.* 1997) and J99 (Alm *et al.* 1999), has demonstrated that both strain genomes are within this size range and both have a G + C composition of 39 % mol (Alm and Noonan 2001). Considerable diversity was identified between the two strains, with 89/1496 and 117/1590 of the putative open reading frames unique to strains J99 and 26695, respectively (Alm and Noonan 2001). Many genotyping-based studies have confirmed this high-level inter-strain variation in *H. pylori* also (Maggi *et al.* 2001;Salaun *et al.* 1998). It is now recognised that the panmictic population structure of *H. pylori* is likely to have arisen by virtue of the fact that these bacteria are often naturally competent and thus transformable, allowing free genetic recombination between strains (Achtman *et al.* 1999;Suerbaum *et al.* 1998).

## 1.3 Epidemiology and transmission of *H. pylori*

*H. pylori* infection has been reported in human populations worldwide. Recent reviews summarising numerous serological studies confirmed that in the developed world, prevalence of *H. pylori* increases with age, with overall reported incidences ranging from 25 - 50 % (Brown 2000;Go 2002;Vaira *et al.* 1998). Studies that compared sera in matched populations collected in the last four decades have demonstrated an overall decline in prevalence of infection, suggesting decreasing rates of infection (Fujisawa *et al.* 1999;Roosendaal *et al.* 1997). In contrast, infection rates in developing countries remain high (50 % - 100 %) (Brown 2000), with the majority of individuals seroconverting early in childhood. These differences probably relate to socio-economic differences, with overcrowded living conditions, poor hygiene and multiple siblings all recognised as potential risk factors for infection (Brown 2000;Go 2002;Vaira *et al.* 1998). In the UK, a recent serological surveillance study demonstrated that approximately 7.5 million people in England and Wales are likely to have active *H. pylori* infection (Vyse *et al.* 2002). Thus, even in developed countries where the incidence is decreasing, *H. pylori* infection remains a significant public health problem.

With the exception of one research group that reported infection in sheep (Dore *et al.* 1999;Dore *et al.* 2001) and reports of induced *H. pylori* infection in some laboratory animals (Go 2002), there has been little evidence to suggest a non-human reservoir for this organism. Studies providing evidence of possible intra-familial transmission (Bamford *et al.* 1993;Dominici *et al.* 1999) as well as reports of higher *H. pylori* prevalence in institutionalised populations (Harris *et al.* 1995;Lambert *et al.* 1995) suggests person to person transmission. The demonstration of a strong correlation between *H. pylori* and hepatitis A virus infection in 434 hospital staff

suggested that transmission occurs by a faecal oral route (Rudi *et al.* 1997) although other studies were unable to support this concordance (Fujisawa *et al.* 1999;Luzza *et al.* 1997). Numerous studies have identified *H. pylori* DNA fragments (Makristathis *et al.* 1998;Makristathis *et al.* 2000;Mapstone *et al.* 1993b;Monteiro *et al.* 2001b) and antigens in stool specimens (Konstantopoulos *et al.* 2001;Makristathis *et al.* 2000;Oderda *et al.* 2000;Vaira *et al.* 1999), but few groups have successfully cultured the organism from stools (Dore *et al.* 2000b;Kelly *et al.* 1994;Thomas *et al.* 1992). One early study of Peruvian children suggested that faecally contaminated water may be a risk factor for infection (Klein *et al.* 1991), a possibility supported by the subsequent demonstration of *H. pylori* DNA in drinking water (Bunn *et al.* 2002;Hulten *et al.* 1996) and viable cells in untreated waste water (Lu *et al.* 2002).

An alternative route of transmission for *H. pylori* is by oral-oral or gastro-oral spread. Evidence of transmission via saliva was provided by studies that identified maternal pre-mastication to feed infants in Western Africa (Megraud 1995b) and use of chopsticks to eat from a communal food dish (Chow *et al.* 1995) as risk factors for infection. While numerous reports have successfully amplified *H. pylori* DNA from saliva and dental plaque (Berroteran *et al.* 2002;Li *et al.* 1996;Namavar *et al.* 1995), culture has proved to be unsuccessful in many cases and so the significance of the oral cavity as a potential source of infection remains unclear. Furthermore, no evidence of a common route of transmission was found for *H. pylori* and Epstein Barr virus, a common infection transmitted in saliva (Luzza *et al.* 1998). However the same group later identified vomiting siblings as a risk factor for infection (Luzza *et al.* 2000). Further evidence that aspiration via vomitus may transmit infection was provided by the demonstration of *H. pylori* by culture and PCR in induced (Parsonnet *et al.* 1999) and naturally occurring vomitus (Leung *et al.* 1999) as well as in the surrounding air

(Parsonnet *et al.* 1999). Further development of tools to facilitate investigations of each of these possibilities will be essential in establishing the route of transmission of *H. pylori*.

## 1.4 Clinical presentation of *H. pylori* infection

All patients infected with *H. pylori* develop chronic gastritis, although the majority remain asymptomatic (Go 2002). Spontaneous clearance of infection once acquired can occur, but is uncommon (Go 2002). For a small proportion of patients, infection will progress to clinically significant disease, including gastric ulcer (GU), duodenal ulcer (DU), B-cell mucosa-associated lymphoid tissue (MALT) lymphoma and gastric carcinoma (GC) (Suerbaum and Michetti 2002). The precise mechanisms of these differential disease outcomes are not well understood, although the distribution of *H. pylori* in the gastric mucosa and the effect of this on acid secretion will influence clinical progression (Calam and Baron 2001). For instance, infections focused in the gastric antrum lead to increased gastrin release by gastric G cells and acid hypersecretion. As a result, protective gastric metaplasia form in the duodenum, allowing colonisation of *H. pylori* and formation of duodenal ulcers. In contrast, more diffuse bacterial colonisation of the gastric corpus can lead to decreased acid secretion by the inflamed gastric parietal cells and to development of gastric ulcers (Calam and Baron 2001). Acid hyposecretion and gastric atrophy are also recognised as potential pre-cursors to development of gastric cancer. The factors involved in these differing disease progressions are poorly defined, but are likely to be related to the individual host (Nguyen *et al.* 1999) as well as to bacterial strain virulence. *H. pylori* virulence factors are considered in more detail in section 1.12.

## 1.5 Diagnosis of *H. pylori* infection

A recent UK survey revealed that 9.0 % of all adults interviewed had consulted their doctor about dyspepsia in the previous year (Logan and Delaney 2001). The approach to management of dyspeptic patients is still a matter of some debate, but testing patients for *H. pylori* infection is a key component of their initial investigation. Furthermore, patient testing as a follow-up after treatment to assess success of eradication therapy is often necessary, particularly in persistently symptomatic patients. A consensus meeting of the European *Helicobacter pylori* Study Group (EHPSG) established European guidelines for the management of *H. pylori* infection (1997). The recommendations were subsequently refined following an update meeting (Malfertheiner *et al.* 2002). A "test and treat" strategy was recommended for persistently dyspeptic adult patients under 45 years of age, where testing was by urea breath test or stool antigen test, described in sections 1.5.2.1 and 1.5.2.3, respectively. There are many tests available for diagnosis of *H. pylori* infection, so selection of an appropriate method requires an appreciation of the relative merits of each approach. Methods of *H. pylori* diagnosis can be subdivided into two main categories, invasive and non-invasive.

### 1.5.1 Invasive diagnostic methods

It has been recommended that at the initial presentation of a symptomatic patient, endoscopy should be conducted to exclude any (pre) malignant disease, particularly in patients > 55 years old or in those with 'alarm' symptoms, such as weight loss or signs of gastrointestinal bleeding (Braden and Caspary 2001). Not only does endoscopy allow macroscopic evaluation of disease, but gastric biopsies can be collected at the same time for examination by one or more of the many tests available

for *H. pylori*. One disadvantage of all invasive tests is the potential sampling error that may arise from the patchy distribution of *H. pylori* in the gastric mucosa (Vaira *et al.* 2002). In addition, any invasive method like endoscopy carries a potential, albeit small, risk of complications for the patient.

#### 1.5.1.1 Histology

Histological analysis of biopsies to identify *H. pylori* is now widely used and has the advantage that it also allows the degree of inflammation and any other associated pathologies to be assessed (El Zimaity 2000). Although histology is considered by some to be the "gold standard" diagnostic method, its sensitivity depends largely on the expertise of the pathologist, the extent of biopsy sampling and the staining method used. It has been recommended that ideally, to maximise sensitivity, three biopsies should be taken from the antrum and from the greater and lesser curvature of the gastric corpus (El Zimaity 2000). Routine haematoxylin and eosin staining is not sufficiently reliable and can miss low-density infections. Various other stains are available that are reported to improve sensitivity, including Warthin-Starry stain, Giemsa and Genta, as well as immunostaining for improved specificity. Although these approaches vary in terms of labour, expense and ease of interpretation, none have been shown to be significantly superior to others. However, Giemsa staining is inexpensive, sensitive and easy to perform (Vaira *et al.* 2002).

#### 1.5.1.2 Culture

Culture provides definitive evidence of infection and so is the most specific of all diagnostic tests. However, as *H. pylori* is a fastidious, slow-growing organism that requires microaerobic conditions, culture is both time-consuming and technically demanding. Sensitivity depends on both the biopsy transport conditions and the expertise of the individual laboratory (Perez-Perez 2000). Many studies have

demonstrated that the composition of transport media used is critical for *H. pylori* survival, with normal saline, Stuart's transport medium and 20 % glucose having been suggested (Vaira *et al.* 2002), while the inclusion of antibiotics in other media (e.g. Dent's supplement) prevents overgrowth of bacterial contaminants from the oral cavity. To date no recommended transport medium is used universally, and this lack of standardisation possibly contributes to inter-laboratory variations in sensitivity of the technique. Additionally, delay (>24 hr) in processing of biopsies significantly decreases bacterial viability and sensitivity (Perez-Perez 2000). In specialised laboratories, sensitivity of culture can be over 90 % (Nakamura 2001), but this can be reduced in patients that have had prior antibiotic therapy, while lowering gastric pH with proton pump inhibitors (PPIs) can lead to overgrowth of contaminating oral bacteria. The major advantage of culture over all other methods is that it provides the means for gaining further strain information that may be key in appropriate patient management, including examination of virulence potential and determination of antibiotic susceptibilities. The importance of these will be discussed in subsequent sections (1.12 and 1.11).

#### 1.5.1.3 Rapid urease test (RUT)

This test is based on the characteristic rapid urease activity of *H. pylori* that splits urea to form ammonia and $CO_2$. The original *Campylobacter*-like organism (CLO) test comprises a calibrated amount of buffered gel containing urea and pH indicator, with bacteriostatic agents to prevent growth of other urease-producing organisms. A biopsy containing *H. pylori* will hydrolyse urea within 1-24 h, resulting in release of ammonia and a change in pH indicator colour from yellow to red. The urease enzyme can denature in an acid environment, so intensity and speed of a positive reaction depends not only on the number of organisms present but also on the thickness of the

protective mucus layer (Midolo and Marshall 2000). Other gel-based tests (e.g. Hpfast) and paper-based tests (e.g. PyloriTek®), based on similar principles are now available (Vaira *et al.* 2002). A major advantage of the RUT is that it is rapid and inexpensive. Pre-treatment, sensitivities can range from 80 – 95 % while specificities range from 95 – 100 % (Vaira *et al.* 2002). Buffered urea tests require a minimum of 1000 organisms to produce a positive result, and false-negatives can also result from previous patient medications such as PPIs, antibiotics and $H_2$ receptor antagonists. Inactivation of *H. pylori* by the raised pH found in the stomachs of achlorhydric patients can lead to falsely negative results also, while lower sensitivities, ranging from 69 – 75 % have been observed in paediatric populations (Midolo and Marshall 2000). Falsely positive RUT results are less common, but can occur in patients taking PPIs whose gastric mucosa may be contaminated with urease-positive oral bacteria (Midolo and Marshall 2000). Therefore this test may not be appropriate for accurate diagnosis in certain patient groups.

### 1.5.2 Non-invasive methods

Diagnostic methods that do not require prior endoscopy have the obvious advantage that they do not incur the considerable associated costs of an invasive approach and they overcome the small, but recognised risk to the patient.

#### 1.5.2.1 Urea breath test

This test also exploits the potent urease activity of *H. pylori*. Urea labelled with $^{14}C$ or $^{13}C$ isotopes is ingested by patients and this is split by *H. pylori* urease, to evolve labelled $CO_2$ that diffuses into the bloodstream and is transported to the lungs. Patient's breath is collected and analysed for either $^{14}CO_2$ or $^{13}CO_2$. An elevated ratio of isotopically labelled $CO_2$ relative to unlabelled $CO_2$ indicates *H. pylori* infection

(Vaira *et al.* 2000). Although lower doses of $^{14}C$ have been administered in children, there are concerns about the use of radioactive isotopes in paediatric patients and in pregnant women. A safer alternative is the non-radioactive isotope $^{13}C$, but detection of this by mass spectrometry restricts its use to specialist centres. A recent review summarising numerous studies showed that UBT was sensitive (90 - 100 %) and specific (86 - 100 %) (Vaira *et al.* 2000), although false-negative results can occur in patients that have had prior antibiotic or PPI therapy. In spite of this, UBT is well-recognised as a rapid and accurate means of non-invasive *H. pylori* diagnosis. Additionally, UBT is particularly suitable for non-invasive follow-up patient testing after specific eradication therapy.

**1.5.2.2 Serology**

*H. pylori* infection leads to local mucosal and systemic immune responses, including raised systemic IgA and IgG antibody levels. This has led to the development of a wide range of specific serology tests for *H. pylori*, many in an enzyme linked immunosorbent assay (ELISA) format as well as western blotting, immunochemistry and complement fixation-based approaches (Ho and Marshall 2000). Serology has been invaluable in epidemiological studies of *H. pylori* infection (Vyse *et al.* 2002). As well as being comparatively inexpensive, one major advantage of serology is that it is less likely to be adversely affected by the patient having had prior PPI or antibiotic therapy. A survey of over 50 PHLS laboratories in England and Wales indicated that serology is widely used as a diagnostic tool in the management of dyspeptic patients (R. J. Owen, personal communication). However several studies have demonstrated that specific antibody can persist for many months or years after resolution or eradication of *H. pylori*. Thus, a major limitation of this approach is that it cannot distinguish between past and active, ongoing infection. For this reason,

serology is unsuitable for monitoring success of eradication therapy (Vaira *et al.* 2002). The accuracy of different serology tests varies considerably, with reported sensitivities and specificities ranging from 70 – 100% and 31 – 100%, respectively (Ho and Marshall 2000). Additionally, the cut-off values of tests often require adjustment for different geographical population groups (Graham and Qureshi 2001), while testing in young children may be unreliable, possibly due to an immature immune response (Kolho *et al.* 2002;Okuda *et al.* 2002). It is thus evident that although convenient, serology is not a reliable method for diagnosing active infection.

**1.5.2.3 Stool antigen test**

The recent development of an ELISA-based kit for detection of specific antigen in stool specimens has been met with considerable interest as it is non-invasive, does not require highly specialised equipment and, unlike serology, is more likely to provide evidence of active, rather than past infection. Furthermore, it may be more appropriate for use in paediatric patients than serology or invasive methods. Two such commercial ELISA kits are now available in the UK, namely the Premier Platinum HpSA kit and the Amplified IDEIA HpStAR kit, (formerly the FemtoLab Cnx *H. pylori* kit). Numerous evaluative studies have been conducted to date, the majority of which have examined the performance of the Premier Platinum HpSA kit as a means of primary diagnosis as well as post-eradication follow-up. A recent review of 43 studies comprising a total of 4769 patients reported that overall the HpSA kit was a highly sensitive (92.4 %) and specific (91.9 %) means of diagnosing infection in untreated patients (Gisbert and Pajares 2001), although performance of this assay was reported to be considerably lower in some individual studies included in this analysis. Analysis of 25 studies examining a total of 2078 post-treatment patients demonstrated that HpSA could also be used as a sensitive (88.3 %) and

specific (92.0 %) follow-up test at ≥ 4 weeks to assess eradication outcome. However, some studies presented evidence contradictory to this (Gisbert and Pajares 2001). Results reported from one of the few studies that have compared the HpSA kit with the newer IDEIA HpStAR kit suggested that the latter is marginally more sensitive (93.8 % *vs.* 98.0 %) for primary diagnosis of paediatric infection (Makristathis *et al.* 2000). Similarly, the IDEIA HpStAR kit was reportedly more specific than the HpSA kit for post treatment follow up of paediatric patients (Makristathis *et al.* 2000). A different study of 148 adult dyspeptic patients demonstrated that the sensitivity of the IDEIA HpStAR kit for follow-up of patients after eradication therapy was higher (88.6 %) than that of the HpSA kit (80.0 %) (Leodolter *et al.* 2002), while a large prospective multi-centre study of 302 symptomatic children showed that the kit was sensitive (97.8 %) and specific (99.0 %) for primary diagnosis, compared with culture, histology and RUT (Koletzko *et al.* 2003). A comparative evaluation of these two kits, as well as a recently-available rapid immunochromatographic test is presented in Chapter 8.

## 1.6 Polymerase Chain Reaction (PCR)

It is evident from the previous section that no single diagnostic method is markedly superior to others. As discussed, while non-invasive methods are preferable, it is culture alone that offers a means of further strain characterisation. However the associated delays as well as the limited availability of this method has prompted the search for alternative molecular methods, to examine the *H. pylori* genome directly from clinical samples. One technique central to these developments has been the polymerase chain reaction (PCR). The utility of this technique in the examination of human *Helicobacter* infections is the focus of the work presented in this thesis.

### 1.6.1 History of PCR

The polymerase chain reaction (PCR) is a sensitive amplification technology that generates many millions of copies of a gene or gene fragment from as little as a single DNA molecule. PCR was devised in 1983 by Kary Mullis, who demonstrated the potential of his hypothesis in a single-tube experiment that amplified a 25-bp fragment of a plasmid. Further experimentation demonstrated that much larger fragments could be amplified almost exponentially (Mullis and Faloona 1987). PCR was a revolutionary development in molecular biology and its potential in medical diagnostics was quickly realised following the first description of PCR-based diagnosis of Sickle Cell anaemia (Saiki *et al.* 1985). PCR is now widely accepted as an invaluable diagnostic tool for numerous medical conditions, as it is not only rapid, but also potentially highly sensitive and specific.

### 1.6.2 The principle of PCR

PCR-based synthesis of specific DNA fragments is usually catalysed by thermo-stable *Taq* polymerase (originally purified from *Thermus aquaticus*), in a reaction mix also containing the following: specific DNA template to be copied, two short oligonucleotides (approximately 20 – 30 bp in length) that prime further strand extension by complementing sequences flanking the specific area or gene of interest, all four dNTPs to build the synthesised strand and $MgCl_2$ that acts as a co-factor for *Taq* polymerase and also alters DNA charges to facilitate primer-template interactions (Clewley 1999). Specific DNA is amplified by a process of thermal cycling, conventionally in a metal block, as summarised in Figure 1.1.

Briefly, double stranded DNA is denatured at 94 °C to form single strands. This enables specific oligonucleotide primers to access and hybridise complementary sequences, when the reaction temperature is lowered to an annealing temperature (Ta)

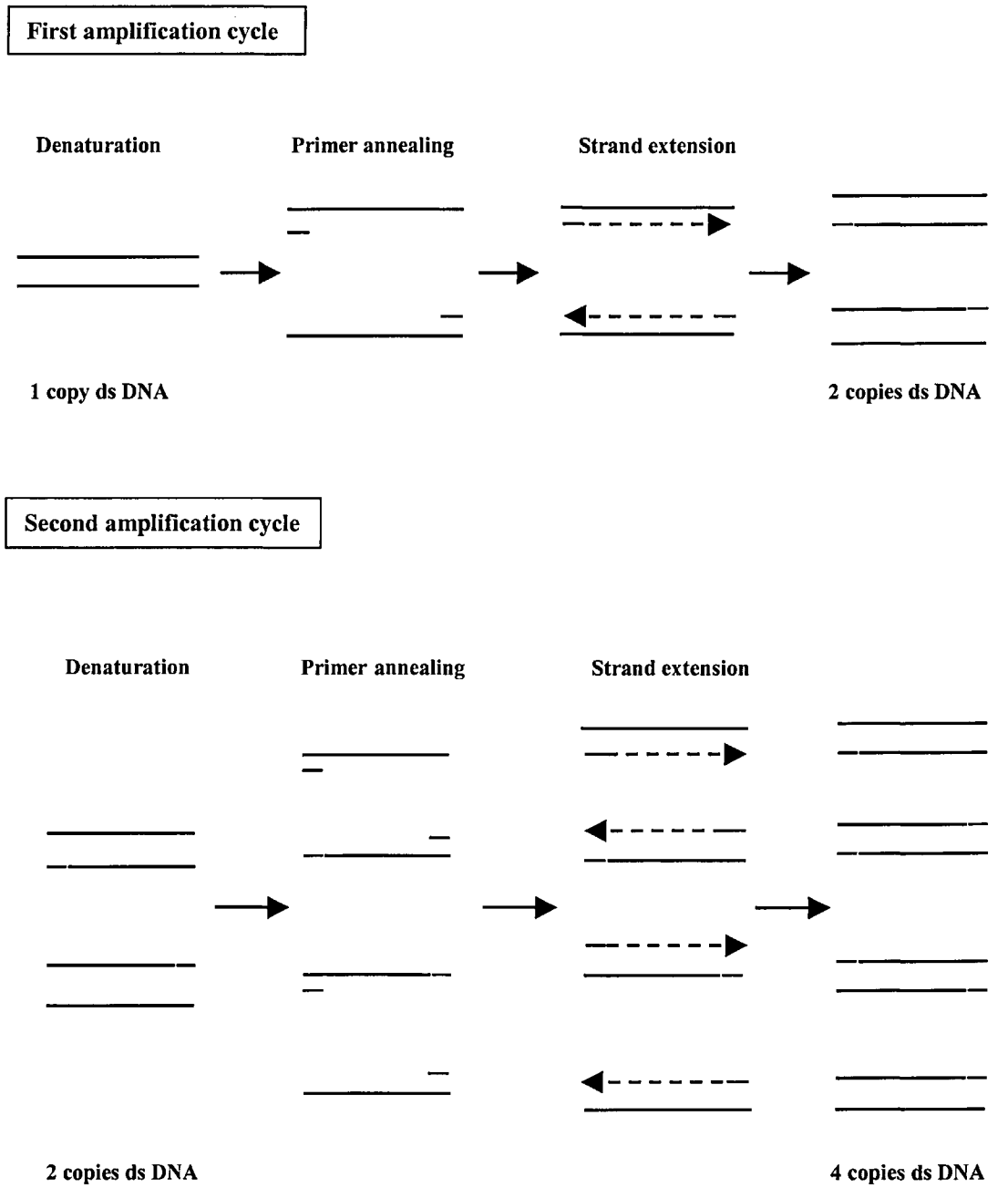



**Figure 1.1: Schematic representation of specific DNA amplification by the Polymerase Chain Reaction (PCR).** Double stranded (ds) DNA is denatured at 95°C and oligonucleotide primers annealed to specific complementary sequences at an appropriate temperature based on the primer Tm. DNA polymerase extends complementary strands and the thermal cycling is repeated, leading to an exponential increase in DNA copy numbers at the end of each cycle.

(typically in the range of 50 – 60°C) that varies according to the melting temperature (Tm) of the individual primer. Sequence immediately downstream of the primer is then extended by *Taq* polymerase at 72 °C by dNTP incorporation. Extension time varies according to the size of the fragment to be amplified, with approximately 35 - 100 nucleotides extended per second at 75 °C (Wittwer and Garling 1991). Cycling of denaturation, annealing and extension temperatures 25 – 50 times leads to exponential increase in copy numbers of the DNA fragment (Figure 1.1). Conventionally, PCR products (amplicons) are visualised by ethidium bromide staining in an agarose gel, following electrophoresis.

### 1.6.3 PCR modifications

The basic format of PCR outlined in section 1.6.2 offers rapid and sensitive DNA amplification. However over the last two decades, many modifications of this format to tailor the methods for specific applications have been described, some of which are considered below.

#### 1.6.3.1 Nested PCR

Although conventional PCR is reported to be highly sensitive, low levels of target may not be amplified to an extent that allows visualisation in an agarose gel. In a nested format, amplicon from the first PCR serves as template for a second amplification reaction involving two novel primers complementary to sequences internal to the first primer set, and so within the template DNA (Figure 1.2). Alternatively one novel primer plus one of the original primers can be used in a hemi-nested format. As specific target DNA levels will have been amplified in the first round of PCR, there will be greater copy numbers of starting template added to the second PCR reaction for further amplification. Thus a major advantage of nested PCR is that it can increase sensitivity of an assay 10 to 1000 fold or more (Bamford *et*

*al.* 1998;Ho *et al.* 1991;Wang *et al.* 1993). Additionally, use of a second pair of primers should improve specificity of a test, theoretically, as four, instead of two, specific primer hybridisations to complementary template sequence are necessary to generate specific product. However, as the potential for contamination of second-round reactions with exogenous amplicons from previous first-round reactions is high, falsely positive results are a potential limitation of this approach, highlighting the need for strict adherence to appropriate preventative measures.

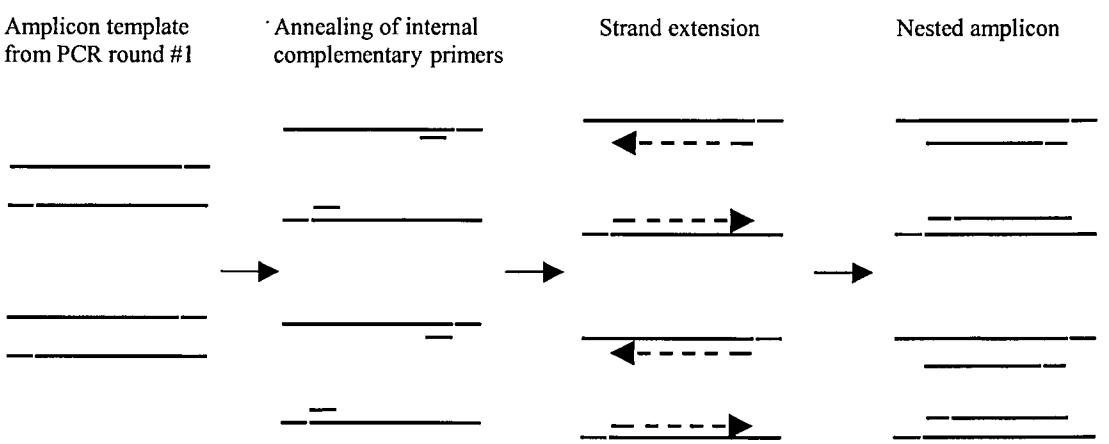



**Figure 1.2 Schematic representation of nested format PCR.**

A second round of PCR is performed where primers anneal to complementary sequences found within amplicon template from a previous PCR. Strands are extended to form copies of DNA fragments internal to the first-round PCR amplicon.

### 1.6.3.2 Multiplex PCR assays

Multiplex PCR is a modification of a conventional PCR assay where, by the inclusion of multiple primer sets, more than one target can be simultaneously amplified in a

single PCR reaction. This approach has the obvious advantage of reducing both reagent costs and labour. Factors that are critical for efficient single-reaction (uniplex) PCR, such as good primer design, adequate, good quality template and ready availability of reagents will also affect the performance of multiplex PCR. However combination of more than one primer pair in a reaction presents some additional problems. Specificity can be reduced by the increased risk of primer dimer formation, due to internal sequence complementarity, leading to spurious amplification. This can deplete the reaction components available and therefore decrease sensitivity of specific target amplification also. In addition, some targets will be preferentially amplified for various reasons, including differences in target fragment or gene length, availability, secondary structure or levels in a sample as well as differences in primer binding efficiency. Although it can be difficult to predict accurately the performance of combined primer sets, ideally those that amplify their respective target with similar efficiency should be selected. This can be predicted by selecting primers with similar annealing temperatures (based on length, and G + C content), and the possibility of primer dimer formation should be minimised also by avoiding sequence homologies (Elnifro *et al.* 2000).

**1.6.3.3 Real-Time PCR**

Conventional PCR can be completed in 2 – 4 hours, but the time taken to visualise amplicons by agarose gel electrophoresis can add considerably (1 – 2 hr) to the total test time. PCR has been revolutionised in recent years by the development of various instruments including the LightCycler (Roche), the Taqman (Applied Biosystems), the Smart Cycler (Cepheid) and the Rotor-Gene (Corbett Research) that monitor amplicon generation in real-time, as the reaction progresses. Although the precise chemistry used varies for individual instruments, typically changes in specific

fluorescent dye emissions are recorded and related to increased levels of amplified DNA. Studies presented in Chapters 5, 7, 8 and 10 examined the potential role of the LightCycler for investigating *H. pylori* infections. A detailed explanation of the principles and applications of the LightCycler is provided in section 2.20.

## 1.7 PCR assays available for detection of *Helicobacter*, and their applications

Numerous PCR detection assays have been described that amplify a range of different specific fragments of the genome of *H. pylori* and other helicobacters. Initially such assays were applied to bacterial cultures and gastric biopsies. Some have been used in the detection of helicobacters from extra-gastric sites also. As PCR-based detection of helicobacters is explored in Chapters 3, 4, 8 and 9, the relative merits of each target gene/fragment and individual PCR assays will be considered in detail.

As illustrated in the following sections, generally many studies report similar levels of sensitivity and specificity for PCR-based detection in gastric biopsies, but detection in oral specimens and faeces would appear to be more variable. Comparison of PCR assay performances for a given application is difficult, as there are numerous variables between studies, including specimen type, sample collection and DNA extraction methods, as well as specific amplification target and primers applied. Individual assay performance may also be affected by inter-laboratory variation in equipment and reagents used. Additionally, most studies examined relatively small numbers of specimens, and patient inclusion often had a selection bias. Many studies of dyspeptic patients contained a disproportionately high number of *H. pylori*-positive individuals in relation to local geographical prevalence, while most studies investigating potential associations between helicobacters and extra-gastric disease include non-dyspeptic patients that are less likely to be *H. pylori*-positive. These aspects will influence the positive and negative predictive values of tests and prevent

accurate determination of assay sensitivities and specificities. A summary of the *H. pylori* specific assays available and their applications is presented in Table 1.2.

### 1.7.1 16S rRNA assays

PCR assays for detection and identification of bacterial species frequently are designed to target ribosomal RNA as this molecule is universally present in all bacterial species, with subunit genes typically arranged in an operon in the order 16S:23S:5S. In contrast 16S and 23S:5S are not adjoining in either of the sequenced strains of *H. pylori* (Alm and Noonan 2001). While some regions of 16S rDNA are highly conserved, others are hypervariable, as illustrated in Figure 1.3. Extensive investigation of 16S rRNA has defined bacterial phylogenetic and evolutionary relationships - analysis of 16S rRNA was central to the creation of the new genus *Helicobacter*, distinct from *Campylobacter* (Goodwin *et al.* 1989). By designing primers that span appropriate regions of this gene, specificity of a PCR assay can be tailored to detect DNA at the prokaryotic, genus or species level. As most bacteria possess multiple copies of rRNA genes, a PCR assay targeting this part of the genome should theoretically be more sensitive. *H. pylori* possesses at least two copies of 16S rRNA and 23S rRNA (Linton *et al.* 1992), although the relative location of these in the genome can vary (Taylor *et al.* 1992). Over 15 000 16S rRNA gene sequences from a diverse range of bacterial species are available in public databases, such as the ribosomal database project (http://www.rdp.cme.mus.edu), facilitating development of specific assays. Furthermore, the 16S rRNA gene is small enough (approx. 1500 bp) for relatively simple sequence determination but large enough to contain sufficient information for speciation and classification purposes. Several *H. pylori* specific PCR assays targeting 16S rRNA have been developed to investigate gastric and extra-gastric *Helicobacter* infections in humans, summarised in Figure 1.3.

**Table 1.2 Summary of the range and application of *H. pylori*-specific PCR detection assays described**

| Gene | Primer pair (N = nested) | Amplicon size | Detection limit (cfu or pg DNA) | Reference | Clinical specimen applications (references in text) |
|---|---|---|---|---|---|
| 16S rRNA | CP1/CP2 | | NS | (Hoshina *et al.* 1990) | Gastric biopsies |
| | Hp1/Hp3 | | 0.1 pg | (Ho *et al.* 1991) | Gastric & appendix biopsies, saliva, dental plaque, stools, buffy coats, atheromatous plaques |
| | Hp1/Hp2 N | 109 bp | 0.01pg | | |
| | JW21/JW22 | 139 bp | NS | (Weiss *et al.* 1994) | Gastric biopsies, stools saliva, vomitus, plaque |
| | | 534 bp | 0.05 – 0.5 cfu | (Wahlfors *et al.* 1995) | Gastric biopsies, dental plaque |
| | ACT-1/ACT-2 | 537 bp | 1 – 25 cfu, 1 fg | (Thoreson *et al.* 1995) | Gastric biopsies |
| | HP1/HP2 RT | 522 bp | 2 cfu | (Engstrand *et al.* 1992) | Gastric biopsies, dental plaque |
| *ureA* | HPU1/HPU2 | 411 bp | 10 – 100 cfu | (Clayton *et al.* 1991) | Gastric biopsies, dental plaque, stools, bile, atheromatous plaques |
| | HPU1/HPU2 | | 1 single cloned | (Wang *et al.* 1993) | Gastric biopsies, atheromatous plaques |
| | Internal primers N | 361 bp | fragment | | |
| | HPU1/HPU2 | | | (Lin *et al.* 1995) | Bile |
| | Internal primers N | 258 bp | | | |
| | HPU1/HPU2 | | | (Mravak-Stipetic *et al.* 1998) | Various sites in oral cavity |
| | HPU1a/HPU2b N | | | | |
| *ureB* | HPU54/HPU18 | | | (Clayton *et al.* 1991) | Gallbladder biopsies |
| | HPU55/HPU17 N | 115 bp | | | |
| *ureC* (=*glmM*) | ureCF/ureCR | 294 bp | 100 pg | (Brisou *et al.* 1990) | Gastric biopsies, saliva, oral ulcer swabs |
| | UreCF/ureCR | | < 10 cfu | (Bamford *et al.* 1998) | Gastric pulmonary and tracheal biopsies |
| | UreCF2/ureCR2 N | 252 bp | | | |
| | GlmMF/glmMR | | 0.1 pg 500 cfu | (Goosen *et al.* 2002) | Gastric biopsies, dental plaque, saliva |
| | glmMF/glmMI N | 496 bp | | | |
| 26 kDa antigen (= *ahpC*) | Sa26F/sa26R | 281 bp | 70 cfu | (Hammar *et al.* 1992) | Gastric biopsies |
| | Sa26F/sa26R | | | (Makristathis *et al.* 1998) | Stools |
| | Sa26F/sa26R2 N | 209 bp | | | |
| 0.86 kb fragment | EHC-U/EHC-L | 417 bp | 0.1 pg | (Li *et al.* 1996) | Gastric biopsies, saliva, stools |
| | EHC-U/EHC-L | | | (Song *et al.* 2000) | Gastric biopsies, dental plaque |
| | ET-5U/ET5L N | | | | |
| 1.9 kb fragment | CAM-2/CAM-4 | | 100 cfu | (Valentine *et al.* 1991) | Gastric biopsies, gastric aspirates |

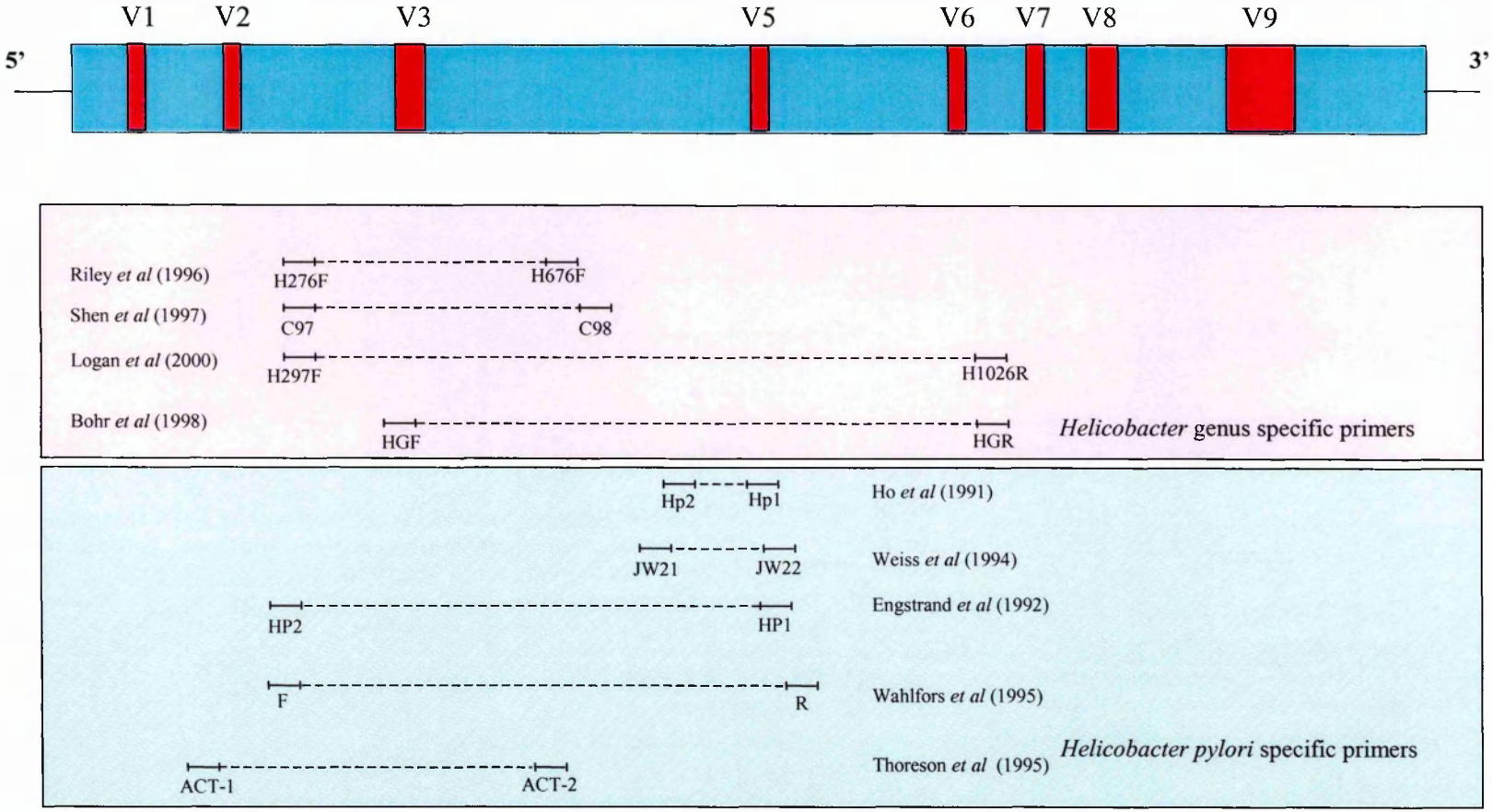



**Figure 1.3** **Schematic representation of the variable regions (V1 – V9) of the *Helicobacter* 16S rRNA gene and relative positions of primers for *Helicobacter* and *H. pylori* specific PCR assays**

#### 1.7.1.1 *Helicobacter* genus specific primers

*Helicobacter* genus-specific primers have been developed and applied in the investigation of helicobacters in extra-gastric disease (section 1.9). Primer pair H276F/H696R that amplifies a 400-bp fragment of 16S rRNA (Figure 1.3) was originally reported to have a detection limit of 5 pg of *Helicobacter* DNA (Riley *et al.* 1996). In contrast, a study investigating the potential bacterial involvement in primary biliary cirrhosis (PBC) demonstrated that these would amplify specific product in *H. pylori* positive gastric biopsies and could detect as little as 10 fg of specific DNA when mixed with liver tissue (Tanaka *et al.* 1999). Very similar to this assay is that described by Shen *et al* (1997). Primer pair C97/C98 amplifies a slightly larger (422 bp) fragment of the same region of the 16S rRNA gene (Figure 1.3). Specificity of this assay was confirmed by the demonstration of successful amplification of DNA from a range of helicobacters but not from other bacterial species (Shen *et al.* 1997). Larger (749 bp) fragments of 16S rDNA were amplified by the primer pair H297F/H1026R, designed and validated as part of a system to distinguish the different bacterial genera of the ε-subclass of the *Proteobacteria* (Logan *et al.* 2000). However as these were not applied for detection purposes, assay sensitivity was not determined. One other assay, using primer pair HGF/HGR amplifies a slightly smaller fragment (639 bp) of the same 16S rDNA region (Figure 1.3). The preliminary report of this assay indicated that it could detect as little as 0.1 pg *Helicobacter* DNA and did not amplify fragments of any of the other bacterial species tested, including genera of the ε-subclass (Bohr *et al.* 1998).

#### 1.7.1.2 *H. pylori*-specific detection PCR assays

The first *H. pylori*-specific PCR assay was developed by Hoshina *et al* in 1990. Examination of gastric biopsies from 10 dyspeptic patients by culture, histology, RUT

and prokaryote as well as *H. pylori* specific PCR demonstrated that all five biopsies positive by conventional tests were also PCR-positive. Three additional biopsies were positive by PCR alone (Hoshina *et al.* 1990). Although it was concluded that PCR was a more sensitive means of *H. pylori* detection, the possibility of poor assay specificity was not excluded.

A different assay, developed by Ho *et al* (1991) (Figure 1.3, Table 1.2) was shown to be 100 % specific, by analysis of bacterial species other than *H. pylori* and a range of human tissue including gastric biopsies from *H. pylori*-negative patients. Application of this to 12 gastric biopsies from *H. pylori*-positive patients generated specific product in all cases (Ho *et al.* 1991). One subsequent study in 1998 identified *H. pylori* by PCR in a significantly higher number of gastric biopsies (116/208) than had been demonstrated by culture and UBT (Oshowo *et al.* 1998). However, while a previous study had generated specific amplicon in 12 gastric biopsies and 12 gastric aspirates of 13 *H. pylori*-positive patients, faint bands were observed in all eight gastric biopsies that were *H pylori*-negative by histology (Mapstone *et al.* 1993a). These results may suggest poor assay specificity. Certainly the reported generation of 109 bp amplicons in a range of human tissues raised the possibility that this assay non-specifically amplified human DNA (Chong *et al.* 1996). However this was not supported by other groups (Leung 1998;Song *et al.* 1999).

An assay developed by Weiss *et al* (1994) (Figure1.3, Table 1.2) generated specific amplicon in 47/50 and 0/45 gastric biopsies from patients that were confirmed *H. pylori* positive or negative, respectively, by a combination of culture, RUT, histology and serology and so was considered 94 % sensitive and 100 % specific (Weiss *et al.* 1994). A further study confirmed this high sensitivity and specificity by successfully

detecting gastric infection in all 11 *H. pylori*-positive patients of the 22 subjects examined in total (Gramley *et al.* 1999).

A different assay, described by Wahlfors *et al* in 1995 (Figure1.3, Table 1.2) amplified specific product in 15 of 29 gastric biopsies, whereas fewer positives had been determined by culture (n = 11), histology (n = 12) or RUT (n = 9). This may indicate that PCR is the most sensitive method, although further tests to confirm amplicon specificity were not conducted.

Sensitivity of an assay, developed by Thoreson *et al* (1995) (Figure 1.3, Table 1.2) when applied to 56 gastric biopsies was reported to be higher than observed for histology but slightly less than that of culture. The assay performance also depended on the method of gastric biopsy DNA extraction. A subsequent study examining 101 gastric biopsies demonstrated that this PCR may be more sensitive than culture and histology combined (Thoreson *et al.* 1999). However, *H. pylori* status was not confirmed by UBT or serology in four of the nine patients that were PCR-positive only, possibly indicating falsely positive results.

One 16S rRNA detection assay that differed from all others was a reverse-transcription PCR (RT-PCR) described by Engstrand *et al.* (1992) (Figure 1.3, Table 1.2). Specific 16S rRNA was first reverse-transcribed to copy DNA (cDNA) for amplification. This assay also amplified 522-bp fragments from other intestinal helicobacters (*H. cinaedi* and *H. mustelae*) and so was not species-specific. Analysis of 15 gastric biopsies demonstrated that the assay was of comparable sensitivity to UBT and culture, but a subsequent study of a larger sample set of 45 gastric biopsies demonstrated a low sensitivity of 47 % compared with UBT (el Zaatari *et al.* 1995).

### 1.7.2 Urease genes

Potent urease activity is a key phenotypic characteristic of *H. pylori* and other gastric *Helicobacter* species, and probably provides protection against the acidic gastric environment. The genes comprising the urease operon (*ure* ABDEFGHI) (Labigne *et al.* 1991) have therefore been a popular target for the development of specific assays for detection of the various gastric helicobacters, including *H. pylori*, *'H. heilmannii'* and *H. felis*. As for 16S rRNA, urease genes have been a preferred target for development of *H. pylori*-specific assays, the more widely used of which will be considered.

#### 1.7.2.1 Urease A

Analysis of 12 bacterial species other than *H. pylori* confirmed that the assay of Clayton *et al* (1991) (Table 1.2) was species-specific. PCR amplicon was generated in 15 of 23 gastric biopsies tested, whereas only ten, seven and three biopsies were positive by histology, culture and urease testing, respectively (Clayton *et al.* 1991;Clayton *et al.* 1992). However, a subsequent study of 66 gastric biopsies, comparing this assay with culture, was unable to demonstrate superior sensitivity of a PCR-based approach (van Zwet *et al.* 1993), while another group that examined 33 formalin fixed gastric biopsies, demonstrated a higher level of positive results with immunohistochemistry (66 %) compared with this PCR assay (45 %) (Ashton-Key *et al.* 1996).

This assay was subsequently modified to develop a nested PCR that increased assay sensitivity 1000 fold and amplified product in 14/17 gastric biopsies tested, ten of which had been positive after the first PCR round. No product was generated in any of the three *H. pylori* negative biopsies although this number of samples is insufficient to determine assay specificity accurately (Wang *et al.* 1993).

### 1.7.2.2 Phosphoglucosamine mutase (*glmM/ureC*)

PCR assays amplifying this target were originally described as targeting the *ureC* gene that was thought to be involved in the urease activity of *H. pylori* and was therefore species-specific. Subsequent studies demonstrated that *ureC* had greater homology with genes that encodes a phosphoglucosamine mutase enzyme that is also found in other bacterial species and the target was renamed *glmM* (De Reuse *et al.* 1997). Details of the assays developed to amplify fragments of this gene are summarised in Table 1.2.

Application of the assay described by Brisou *et al* (1990) to 62 gastric biopsies from 14 patients demonstrated comparable sensitivity and specificity to that of culture (Bickley *et al.* 1993). Further examination of 104 gastric biopsies by culture, histology, RUT and PCR demonstrated that the latter was 100 % sensitive and 97 % specific, using culture as the gold standard (Lage *et al.* 1995). Modification of this assay to a nested format and application of this to gastric biopsies, oral fluids, dental plaque and faeces seeded with *H. pylori* cells, demonstrated a 10 to 1000 fold increase in sensitivity, depending on the specimen type (Bamford *et al.* 1998).

Initial evaluation of another assay, developed by Goosen *et al* (2002), demonstrated specific amplification in 12 isolates of *H. pylori* but not in any of the 31 other bacterial species tested. Performance of this test on gastric biopsies was not evaluated.

### 1.7.3 26 kDa antigen assays

In 1991, O'Toole and colleagues isolated a protein of 26 kDa that was abundant in *H. pylori*. Subsequent cloning of the gene encoding this protein and hybridisation analyses suggested that it was species-specific (O'Toole *et al.* 1991). A later study demonstrated by complementation experiments that this antigen had alkyl

hydroperoxide reductase (AhpC) activity (Lundstrom and Bolin 2000). This protein is discussed further with respect to metronidazole resistance in section 1.11.6.3. A PCR assay amplifying a fragment of this gene was developed by Hammar *et al* (1992) (Table 1.2). Application of this to gastric biopsies of 27 patients indicated that it was as sensitive as culture, but four additional positive results, three of which were not corroborated by high anti-*H. pylori* antibody titres suggested that this assay may lack specificity, although amplicon specificity was confirmed by probe hybridisation also.

### 1.7.4 0.86 kb cloned fragment

A 0.86 kb cloned DNA segment had been characterised previously and proposed to be *H. pylori*-specific by Li and colleagues in 1996. Application of the subsequent assay developed (Table 1.2) to 88 gastric biopsies, generated product in all 71 specimens that were *H. pylori*-positive by histology and in none of the 17 *H. pylori*-negative patient samples (100 % sensitive and specific).

### 1.7.5 1.9 kb fragments

The observation that a cloned 1.9 kb fragment would hybridise with *H. pylori* strains but not with 306 other clinical isolates led to development of an *H. pylori*-specific assay (Valentine *et al.* 1991), (Table 1.2). Analysis of 33 gastric biopsies demonstrated a sensitivity of 93 % and specificity of 100 %, compared with culture and histology. Sensitivity of PCR-based detection was lower (85 %) for matched gastric aspirates.

## 1.8 Non-invasive PCR-based detection of *H. pylori*

While most of the assays discussed thus far allowed sensitive and specific detection of *H. pylori* in gastric biopsies, the intrinsic disadvantages of an invasive approach remain. For this reason, numerous studies have examined the potential of PCR for

non-invasive diagnosis of *H. pylori* gastric infection by specific detection in specimens either collected from the oral cavity or in stools. The PCR assays applied to these specimen types are summarised in Table 1.2.

### 1.8.1 PCR-based detection of *H. pylori* from the oral cavity

Numerous studies have applied PCR in the detection of *H. pylori* from oral specimens (e.g. saliva and dental plaque), but the success of this has varied considerably. This is likely to relate to the sensitivities and specificities of the individual PCR assays as well as to the methods of specimen collection, transport and DNA extraction. The latter factor is particularly critical in dental plaque specimens, that contain high levels of DNA from other bacterial species as well as substances inhibitory to the PCR reaction. Furthermore, differences in study populations such as *H. pylori* prevalence and oral hygiene are likely to influence test outcome.

Some studies were unable to detect specific DNA in oral specimens by PCR assays targeting 16S rRNA (Wahlfors *et al.* 1995) or *glmM* genes (Bickley *et al.* 1993), while primers Hp1 and Hp2 of Ho *et al* (1991) amplified 16S rDNA in oral samples from only 15 of 116 *H. pylori*-positive patients (Oshowo *et al.* 1998). In contrast a much higher rate of *H. pylori* carriage in the oral cavity (85 %) and fingernails (58 %) was demonstrated in a study that applied the nested version of this PCR assay to plaque samples from 242 subjects in Guatemala, although amplicon specificity was not confirmed by any other method (Dowsett *et al.* 1999). The same assay previously was reported to amplify specific product in at least one oral sample (saliva or plaque) for five of 13 *H. pylori*-positive patients (Mapstone *et al.* 1993a).

The RT-PCR assay of Engstrand *et al* amplified specific DNA in dental plaque of 7/18 *H. pylori*-positive patients. All amplicons were confirmed by specific probe

hybridisation, while *H. pylori* was confirmed in two plaque samples by indirect immunofluorescence also (Nguyen *et al.* 1993).

A high rate of detection was reported by a modification of the assay of Li *et al* (1995), involving a second nested round of PCR with primers ET-5U and ET-5L (Song *et al.* 2000). *H. pylori* DNA was amplified in 97 % of dental plaque samples from 41 patients, in spite of a lower rate of gastric infection (26 %) indicated by UBT. Specificity of PCR product was confirmed by Southern hybridisation, however these discrepant results may indicate sample contamination, a potential problem with nested PCR. Similarly, a study using the assay of Clayton *et al* (1991) amplified *ureA* DNA fragments in matched gastric biopsies and dental plaque samples from seven patients, but also in dental plaque from five other patients that had no PCR-based evidence of gastric infection (Berroteran *et al.* 2002).

A lower rate of detection was reported for a study investigating oral specimens of clinically healthy volunteers by a *glmM* PCR assay (Goosen *et al.* 2002). Specific amplicons were generated in only 2/58 specimens, while the primer pair of Clayton *et al*, amplifying *ureB* generated product in 3/58 patients. Sequencing of *ureB* amplicons confirmed the specificity of the novel *glmM* assay and identified one falsely positive *ureB* result, due to amplification of *Staphylococcus epidermidis* DNA.

### 1.8.2 PCR-based detection of *H. pylori* in stool samples

The possibility that stools could be examined for evidence of gastric *H. pylori* infection was first indicated by a report that successfully cultured the organism from the stools of Gambian patients (Thomas *et al.* 1992). However the limited success of this approach (Dore *et al.* 2000b;Kelly *et al.* 1994) suggests that the presence of viable bacterial cells in patient stools may be transient, and variable between different patient groups. These limitations have provided the impetus for development of

alternative detection methods. As discussed (section 1.5.2.3) stool antigen testing is one approach that has proven to be both sensitive and specific, but does not allow any further strain characterisation. Numerous studies have described PCR-based detection of *H. pylori* in stools, with varying degrees of success. Similarly to dental plaque, stool specimens contain high levels of competing bacterial DNA as well as substances inhibitory to PCR. Thus the DNA extraction method is as critical to test performance as the individual PCR assay applied. These aspects are examined in the study presented in Chapter 8.

The *ureA* assay described by Clayton *et al* in 1992 was shown to detect 2.5 x $10^4$ cells per g faeces in artificially seeded specimens and in 11/17 clinical stool samples. Faecal DNA had been extracted by an immunomagnetic separation (IMS) technique whereby *H. pylori* cells are captured by magnetic beads coated in specific antibody and separated from other bacteria and inhibitors (Nilsson *et al.* 1996). Provided the antibody used does not cross-react with other bacterial species, IMS has the potential to improve assay specificity as well as concentrating specific DNA, if *H. pylori* cells are intact in stools specimens. However the lack of success of culture suggests that many *H. pylori* cells are fragmented at this site, and so IMS may decrease sensitivity by capturing specific antigen but not DNA. Nevertheless, a larger study that extracted DNA by an IMS-based method also, combined with the same PCR assay, amplified specific DNA in 61.4 % of faecal samples from 57 patients that had evidence of gastric *H. pylori* infection, but not in 15 uninfected patients (specificity 100 %) (Watanabe *et al.* 1998). Similarly, a different IMS-PCR approach to investigate faecal samples from 47 *H. pylori*-positive and 57 *H. pylori*-negative patients demonstrated 100 % specificity and also higher sensitivity (80.9 %) (Monteiro *et al.* 2001a). A different study that extracted DNA by another method (using Tri reagent)

reported 100 % sensitivity of the Clayton assay, with amplicons generated in all 30 faecal samples from patients that had gastric evidence of *H. pylori* infection also (Russo *et al.* 1999). Amplicon identity was confirmed by *Hinf*I restriction enzyme digestion. The same PCR assay has also been applied in the evaluation of different methods for faecal DNA extraction, including an aqueous two-phase system (Lantz *et al.* 2000) and macroporous polypropylene filters (Cavallini *et al.* 2000) for removal of PCR inhibitors.

Application of the Ho *et al* assay to DNA extracted from faecal samples by a cetyltrimethyl ammonium bromide (CTAB) method, to remove inhibitory polysaccharides, amplified 16S rDNA fragments in 28 of 31 patients with gastritis but not in any of the 11 patients with histologically normal gastric biopsies (Mapstone *et al.* 1993b). In contrast, single round PCR with the Ho *et al* primers Hp1 and Hp2 failed to amplify product in faecal samples from 24 *H. pylori*-positive patients, although specific detection in artificially seeded faeces was possible (van Zwet *et al.* 1994). A different study that used a relatively complex DNA extraction method involving CTAB as well as polyvinylpyrrolidone (PVP) treatment, to remove inhibitory substances, detected specific 16S rDNA fragments by the Weiss assay in 8/11 stools from *H. pylori*-positive patients (Gramley *et al.* 1999). None of the 11 stools from uninfected patients were PCR-positive. An alternative approach was described where faecal DNA was extracted by a comparatively simple method and then specific DNA captured by a biotinylated probe prior to analysis by the Weiss PCR assay (Shuber *et al.* 2002). Application of this to 25 patients, 11 of whom were *H. pylori*-positive, demonstrated high sensitivity and specificity (100 %). Additional analyses of three of these patients suggested that this system was suitable for patient follow-up after therapy also. The Weiss assay was also applied in a study

investigating transmission of *H. pylori* (Parsonnet *et al.* 1999), that amplified specific product in induced stools, post emesis saliva and vomitus specimens of, respectively, 11/16, 9/16 and 16/16 *H. pylori*-positive asymptomatic patients. All samples had been extracted by an IMS-based method. Unusually, specificity of this approach was also confirmed by culture in many of these induced samples.

Less successful was an approach described by Li *et al* in 1996, that amplified fragments of 0.86 kb *H. pylori* DNA, extracted from stools by a CTAB-based method, from only 15/61 infected patients and in 1/10 uninfected patients tested. In contrast, development of the assay of Hammar *et al* (1992) to a hemi-nested assay using a novel reverse primer allowed sensitive and specific *H. pylori* detection from stool specimens extracted by a complex multiple-step method that included adsorption of nucleic acids on a commercial chromatography column containing silica (QIAgen) (Makristathis *et al.* 1998). Specific *ahpC* (26 kDa antigen) fragments were generated in 59/63 and 0/37 samples from *H. pylori* positive and negative patients, respectively. Further testing of patients post-eradication demonstrated that PCR may not be suitable for patient follow-up within one month. Subsequent study of a paediatric population (n = 49) also demonstrated the high sensitivity of this assay (93.0 %), although no negative patients were included to assess specificity (Makristathis *et al.* 2000). However patient follow-up post-eradication demonstrated that false positive PCR results could persist for up to eight weeks.

## 1.9 PCR-based detection of helicobacters in the investigation of extra-gastric diseases

The potential role of helicobacters in various extra-gastric chronic inflammatory conditions has been the focus of increasing numbers of studies. Given the fastidious

nature of many species of *Helicobacter*, PCR has been central to these investigations. This thesis presents a PCR based analysis of four such inflammatory conditions of the bladder, colon and bronchi (Chapter 9).

### 1.9.1 Oral disease

The possibility that helicobacters are in the oral cavity has led to a number of groups investigating the potential contribution of these to inflammatory oral diseases. Development of a nested assay using the Clayton assay in the first PCR round and primers HPU1b and HPU2b in the second round amplified DNA from at least one of seven oral sites in 21/161 patients with oral disease and in none of the 20 controls (Mravak-Stipetic *et al.* 1998). However no associations between oral disease and patient *H. pylori* status or ulcerated versus non-ulcerated lesions and bacterial presence were identified. Similarly, application of the Brisou *glmM* assay to oral ulcer swabs, dental plaque and saliva, demonstrated specific DNA in 11 of 16 ulcer swabs from patients with oral aphthous ulcers and in none of the 23 control patients (Birek *et al.* 1999). However it is difficult to ascribe potential associations between *H. pylori* and recurrent oral ulcers as no further confirmation of amplicon specificity was performed, and *H. pylori* status of the patients was unknown. Furthermore, non-specific amplicons of approximately 294 bp were generated from some strains of *Actinomyces naeslundii*, which is frequently found in the oral cavity. Another study that used the Weiss primer JW22 along with novel primer JW23 (Table 1.2), reported amplicon generation, confirmed by specific probe hybridisation and sequencing, in 24/73 plaque samples collected from 29 periodontitis patients (Riggio and Lennon 1999). More recently, *H. pylori* DNA was amplified in both diseased and healthy areas of the oral cavity of 14 of 36 patients with recurrent oral stomatitis, and in 16 of the 48 control patient samples. The significance of this cannot be interpreted, as

specificity and sensitivity of the novel nested PCR assay applied, amplifying a fragment of the sequenced strain 26695, had not been evaluated (Victoria *et al.* 2003).

### 1.9.2 Diseases of the hepatobiliary tract

Associations are reported between several *Helicobacter* infections and hepatitis in animals (Fox *et al.* 1994;Fox *et al.* 1995;Stanley *et al.* 1994). Consequently, a number of studies have investigated the potential association between helicobacters and human liver and biliary tract diseases.

*H. pylori* DNA in bile was demonstrated by Lin *et al* (1995) for the first time by a novel nested PCR assay (Table 1.2) developed by the authors that generated *ureA* fragments in three of seven bile samples from patients with biliary tract disease. Although specificity of this assay had not been fully validated, partial amplicon sequencing (87 bp) was reported to confirm specificity, despite observed mismatches with a published *ureA* sequence. Similarly, a subsequent study provided PCR-based evidence of helicobacters in the hepatobiliary tract (Roe *et al.* 1999). The Clayton *H. pylori* specific assay (Table 1.2) amplified 411-bp fragments in 12 of 32 bile samples from biliary tract disease patients. DNA was amplified in ten bile samples also by the genus-specific assay of Riley *et al* (section 1.7.1.1), but the authors did not state if these results were corroborative, from the same patient samples, nor was the specificity of the amplicon confirmed (Roe *et al*, 1999). In contrast, the assay described by Shen *et al* (section 1.7.1.1) identified possible presence of helicobacters in 13 of 23 bile samples and 9 of 23 gall bladder specimens, collected from a total of 46 Chilean cholecystis patients (Fox *et al.* 1998). Subsequent amplification of larger amplicons (1200 bp) using primer C97 and a novel reverse primer (C05) allowed sequence characterisation of PCR products from seven bile and four gall bladder samples. This analysis indicated that sequences were most similar to *H. bilis*,

"*Flexispira rappini*" and *H. pullorum*. A different study amplified a specific fragment of *ureB* from the gallbladder mucosa of a single patient, using the assay of Clayton *et al* (1991) (Table 1.2). Although sensitivity and specificity of this assay has not been extensively evaluated, PCR results were validated also by histological evidence of *H. pylori* at this site (Kawaguchi *et al.* 1996), providing compelling confirmatory evidence that *H. pylori* can be found in the biliary tract. However, the Shen assay failed to amplify any specific DNA in bile samples from a larger population of 125 Canadian patients with a range of hepatobiliary conditions (Fallone *et al.* 2003), possibly due to geographical differences in prevalence of bile-resistant helicobacters. Further evidence that these may be less prevalent in the bile of patients from Western countries is provided by a Dutch study that amplified 16S rDNA fragments in only one of 31 patients (Roosendaal *et al.* 2002). A novel PCR assay had been applied that was reported to be of comparable sensitivity to those applied in the study of Fox and colleagues in 1998.

Additionally, evidence suggests that helicobacters may also be found in the liver. One study of 55 liver specimens, 29 of which were from primary biliary cirrhosis (PBC) patients, amplified *Helicobacter* DNA by the Riley assay (section 1.7.1.1) in a single PBC patient only. Amplicon specificity was confirmed subsequently by sequencing (Tanaka *et al.* 1999). In a later study, the Shen assay (section 1.7.1.1) amplified *Helicobacter* DNA in 20 of 24 liver samples from patients with PBC or primary sclerosing cholangitis (PSC), but in only one of the 23 samples from non cholestatic liver disease (NCLD) or control patients. Nine of the positive samples were also positive for at least one of the two specific assays of Thoreson or Hammar, targeting 16S rRNA or *ahpC* fragments of the *H. pylori* genome, respectively. Sequencing of four *Helicobacter*-specific amplicons demonstrated >98 % identity

with *H. pylori* and *Helicobacter* spp. liver 16S rRNA sequences (Nilsson *et al.* 2000). Similarly, the Shen primers amplified DNA in nine of 15 liver tissue samples from Chinese patients with hepatocellular carcinoma and in none of the 13 control samples. Amplicon specificity was confirmed not only by probe hybridisation, but direct sequence analyses demonstrated that these were 99 % similar to 16S rRNA of *H. pylori* and *Helicobacter* spp. liver sequences (Fan *et al.* 2002)

### 1.9.3 Cardiovascular disease

Several serological studies have suggested that *H. pylori* infection could be a risk factor for chronic heart disease (CHD), particularly for the two major types of CHD - atherosclerosis and myocardial infarction. However a meta-analysis of 28 studies (Pellicano *et al.* 1999), including full papers and abstracts showed that studies varied considerably in terms of patient and control selection criteria, methods of *H. pylori* detection and consideration of other co-factors. Although the analysis suggested a weak association between *H. pylori* infection and CHD, study heterogeneity highlighted the difficulty in demonstrating this clearly. PCR has been applied in a small number of studies as an alternative means of investigating the role of helicobacters in this condition.

The first demonstration of *H. pylori* DNA in the cardiovascular system was provided by a study investigating potential association between this infection and coronary atherosclerosis (Danesh *et al.* 1999). Amplicons, subsequently validated by specific probe hybridisation, were generated by the nested Ho assay (Table 1.2) in 1/77 buffy coat samples and 1/39 atheromatous specimens. However, the authors were unable to fully exclude the possibility of specimen contamination. Similarly, *H. pylori ureA* fragments were amplified by Clayton's assay (Table 1.2) in 17/46 atherosclerotic plaques from patients with ischaemic vascular disease (Farsak *et al.*

2000). Specificity of amplification was confirmed by PCR analysis with the Ho *et al* 16S rRNA assay (Table 1.2), but no further amplicon analysis was conducted. In contrast, the assay of Wang *et al* (Table 1.2) was unable to amplify *H. pylori* DNA from atherosclerotic plaques of 51 patients undergoing aortic aneurysm surgery, in spite of the enhanced sensitivity of a nested PCR format (Blasi *et al.* 1996). Similarly, a recent study of atherosclerotic plaques from 32 chronic atherosclerosis patients was unable to amplify *H. pylori* DNA by either of the two *Helicobacter* genus-specific or the two *H. pylori*-specific PCR assays applied, despite high seroprevalence (72 %) in this group (Dore *et al.* 2003).

### 1.9.4 Lower intestine

As discussed in section 1.8.2, numerous studies have detected *H. pylori* by PCR in stool specimens, to allow diagnosis of gastric infection. However few studies have examined the potential role of helicobacters in inflammatory conditions of the lower intestine. One study that examined ten inflamed appendix biopsies by the nested 16S rRNA assay of Ho *et al* (Table 1.2) was unable to provide any evidence of *H. pylori* colonisation or an association with appendicitis (Fanning *et al.* 1998). In contrast, another PCR-based study reported helicobacter DNA from the colon of Crohn's disease (CD) patients by universal amplification of prokaryotic 16S rRNA followed by *Helicobacter*-specific probe hybridisation (Tiveljung *et al.* 1999). Specific examination of colonic tissue of patients with inflammatory bowel disease (IBD) for helicobacters was not described until late in 2002. A novel 16S rRNA *Helicobacter* genus-specific assay using modifications of primers C97 and HGR (section 1.7.1.1) amplified fragments of DNA from four intestinal biopsies, two of which were from CD patients. Sequence based analyses identified amplicons as *H. pylori* in three patients and *H. pullorum* in the remaining sample (Bohr *et al.* 2002).

### 1.9.5 Sudden Infant Death Syndrome

The nested assay of Bamford *et al* was later applied in the investigation of the potential role of *H. pylori* in Sudden Infant Death Syndrome (SIDS) (Kerr *et al.* 2000). Specificity of PCR products generated was confirmed by specific probe hybridisation in an ELISA format, as an alternative to gel electrophoresis and Southern blotting. This approach identified *H. pylori* DNA in at least one of the tissue types (stomach, lungs or trachea) from 25 of the 28 SIDS cases examined and in one of the eight control patients. PCR-based detection of the *cagA* gene was also reported in 25/28 SIDS cases and 1/8 controls. However, only 19 SIDS cases were positive by both assays. This may suggest non-specific amplification in some samples, particularly as the *cagA* gene is not present in all *H. pylori* (Xiang *et al.* 1995). Additionally, other studies were unable to substantiate these findings (Ho *et al.* 2001).

## 1.10 Treatment of *H. pylori* infections

*H. pylori* eradication therapy is strongly recommended in patients with PUD or MALT lymphoma, in patients with signs of gastric atrophy and in relatives of gastric carcinoma patients. Treatment can improve symptoms in ≤10 % of patients with non-ulcer dyspepsia (NUD) and may be advisable in patients with gastro-oesophageal reflux disease (GERD) who are on long-term acid suppressive therapy (Malfertheiner *et al.* 2002). Ideally, treatment regimens should be of short duration (no more than 7 days), should achieve a high cure rate (>90 %) and induce minimal bacterial antibiotic resistance and patient side-effects (Tytgat 1997). Although many antibiotics are active against *H. pylori in vitro*, few are available for treatment due to problems of *in vivo* drug bioavailability. The agents most commonly used in anti-*H. pylori* regimes

include metronidazole (MTZ), clarithromycin (CLA), amoxycillin (AMO), tetracycline (TET) and bismuth salts (BIS). Monotherapy has been shown to achieve poor eradication rates due to development of resistance as well as poor activity of some drugs (e.g. CLA) in the acid gastric environment (Goodwin 1997;Tytgat 1997). The inclusion of agents such as PPIs (e.g. omeprazole, lansoprazole) or $H_2$ receptor antagonists (e.g. ranitidine) to increase gastric pH improves efficacy of acid-sensitive drugs. In addition PPIs may have some anti-bacterial properties and improve bioavailability of AMO by reducing gastric juice volume (Tytgat 1997). However dual therapy still fails to achieve high cure rates. In contrast triple therapies comprising two antibiotics and a PPI or even quadruple therapy regimens that also include a bismuth compound have been shown in clinical trials to achieve high-level eradication rates ranging from approximately 88 – 98 % (de Boer and Tytgat 1996;Qasim and O'Morain 2002).

### 1.10.1 Current treatment recommendations

One other important development from the Maastricht consensus meeting, described earlier (section 1.5) was the issue of guidelines concerning eradication therapy, summarised in Figure 1.4.

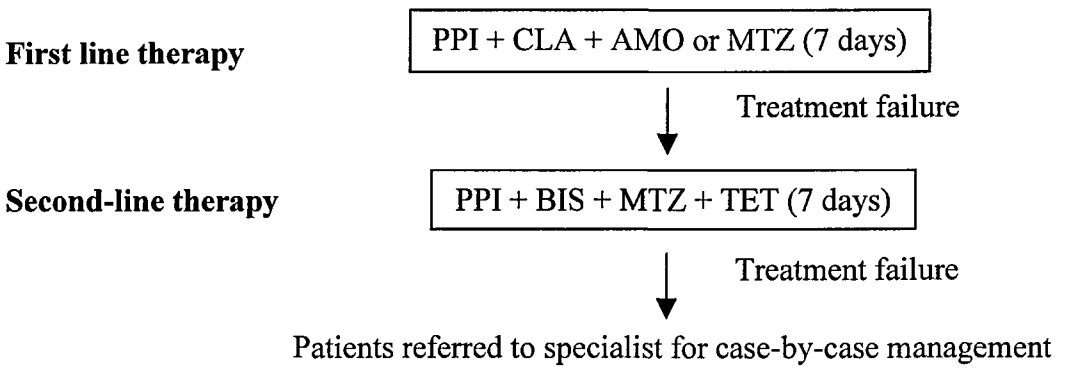



**Figure 1.4 Summary of the recommended eradication strategy for *H. pylori*** (adapted from the Maastricht 2-2000 Consensus Report)

### 1.10.2 Treatment failure

Although triple and quadruple therapies can achieve high rates of eradication, *H. pylori* can persist in as many as 12 % of patients following treatment (de Boer and Tytgat 1996). This may be partially attributable to the physician's reportedly poor adherence to recommended treatment regimens in the primary care setting (Lee *et al.* 2000). Patient compliance with therapy is also likely to be an important contributing factor in treatment failure, although the significance of this is less evident with more potent antibiotic regimens (Tytgat 1997). The recommended EHPSG guidelines of reduced therapy duration of 7 days are likely to improve patient compliance. The positive effects of enhanced patient compliance programmes also have been well-demonstrated (Lee *et al.* 1999). One other factor that has a significant impact on treatment outcome is antibiotic resistance, considered below.

## 1.11 Antibiotic resistance in *H. pylori*

A vast amount of data from numerous studies is available regarding the prevalence of antibiotic resistance in *H. pylori* to the key therapeutic drugs in different geographical areas. Such studies are essential as they provide invaluable information that could direct appropriate therapy. However the current lack of inter-laboratory standardisation of antibiotic susceptibility testing methods can complicate direct comparisons of individual studies. Multiple-centre surveillance studies of antibiotic susceptibilities allow more accurate comparison of resistance levels between states and countries.

### 1.11.1 Clarithromycin resistance

A multinational trial conducted in Europe that examined 485 isolates collected from 47 centres in six west European countries demonstrated CLA resistance rates ranging from 1 to 5 % (Megraud *et al.* 1999). A similar study examining 1274 isolates collected from 22 centres in 17 European countries reported resistance rates ranging from 0 % to over 23 % in Italy, Belgium and Austria (Glupczynski *et al.* 2001). A recent meta-analysis of 20 nationwide trials conducted in the USA that included data from over 3600 patients demonstrated overall CLA resistance levels of 10.1% (Meyer *et al.* 2002).

A meta-analysis of 12 studies comprising a total of 501 isolates, including 33 that were CLA resistant, suggested that although the effects varied between individual studies, overall CLA resistance reduced treatment efficacy by an average of 55 % (Dore *et al.* 2000a). This, combined with high reported rates of CLA resistance highlights the importance of enhanced surveillance of antibiotic resistance in *H. pylori*, not only for individual patient management but also to influence local and national prescribing policies. As discussed (section 1.11), accurate surveillance of

antibiotic resistance is hindered by poor inter-laboratory standardisation. Furthermore, culture-based methods such as disk diffusion, E-test or agar dilution require laboratory expertise, are laborious and can take up to two weeks to obtain a result from the initial receipt of gastric biopsy. PCR-based tests and other molecular tests, such as specific probe hybridisation allow rapid, potentially simple determination of antibiotic susceptibility.

### 1.11.2 The mechanism of CLA resistance

CLA belongs to the class of antibiotics known as the macrolides; bacteriostatic agents that interact with the 50S ribosome and inhibit polypeptide elongation. Areas of the peptidyltransferase loop in domain V of bacterial 23S rRNA are proposed to bind the macrolide, while mutation in this region prevents this interaction, leading to bacterial resistance (Weisblum 1995).

A study that examined this region of 23S rRNA sequence from four pairs of CLA sensitive (CLA-S) and CLA resistant (CLA-R) *H. pylori*, recovered before and after eradication therapy, respectively, demonstrated single point mutations. Sequence analysis of 12 CLA-R isolates in total showed that all strains had adenine to guanine transition mutations at positions 2058 or 2059, according to the *Escherichia coli* numbering system (Versalovic *et al.* 1996), later numbered 2142 and 2143 (Taylor *et al.* 1997). Further analysis of this region of the 23S rRNA gene in three CLA-R isolates demonstrated that A2142C transversion mutations can confer CLA resistance also (Stone *et al.* 1996).

### 1.11.3 Molecular tests for CLA susceptibility testing

As a more economical alternative to DNA sequencing, several PCR-based methods were developed to facilitate collation of data concerning the relative incidences of each mutation type. All studies reported that adenine to guanine mutations (A2142G)

and (A2143G) were significantly more frequent than the transversion mutation to cytosine (A2142C). Some reports, particularly those from Asian countries, indicated that A2143G is identified more frequently than A2142G (Dzierzanowska-Fangrat *et al.* 2001;Maeda *et al.* 1998b;Maeda *et al.* 2000;Pan *et al.* 2002;Sevin *et al.* 1998), but evidence suggests that the latter mutation may be associated with higher CLA MICs (Alarcon *et al.* 2000;Dzierzanowska-Fangrat *et al.* 2001;Marais *et al.* 1999;Piana *et al.* 2002;Pina *et al.* 1998;Stone *et al.* 1997;Versalovic *et al.* 1997).

### 1.11.3.1 PCR-Restriction Fragment Length Polymorphism (RFLP) assays

One approach that has been widely used for CLA susceptibility testing is PCR followed by RFLP. Fragments of 23S rDNA, amplified by PCR, are digested by restriction enzymes such as *Mbo*II (or *Bbs*I) and *Bsa*I (or *Alw*26I) that recognise and cut DNA at specific target sequences created by A2142G or A2143G mutations, respectively (Versalovic *et al.* 1997). This approach has been applied to clinical isolates (Occhialini *et al.* 1997;Dzierzanowska-Fangrat *et al.* 2001;Hulten *et al.* 1997;Piana *et al.* 2002;Szczebara *et al.* 1997;Wolle *et al.* 2002;Yang *et al.* 2001) and later to gastric biopsies (Bjorkholm *et al.* 1998;Sevin *et al.* 1998) and gastric aspirates (Maeda *et al.* 1998b). While PCR-RFLP is faster than culture-based susceptibility testing, one disadvantage was that most studies could not identify all CLA-R isolates as there was not a suitable assay available to detect the A2142C mutation. That problem has since been rectified, with the recent description of such an assay (Menard *et al.* 2002).

### 1.11.3.2 Primer mismatch PCR-based assays

The failure of PCR-RFLP to detect the A2142C mutation led to the development of a primer mismatch PCR assay. This is based on the principle that PCR can be optimised so that if template sequence is not complementary to the last base at the 3'

end of a primer, initiation of strand extension and amplification is prevented. The design of a primer that would only complement with template DNA containing mutation A2142C meant that only isolates with this sequence would be amplified. This approach successfully identified A2142C mutation in five CLA-R isolates that were shown by PCR-RFLP to lack either A to G mutation (Alarcon *et al.* 2000). Two additional assays were later designed to detect both A to G mutations also, and this approach successfully identified A2143G mutations in 5 of 96 clinical isolates tested. All results were confirmed by culture-based susceptibility testing (Pan *et al.* 2002). Although this approach can be performed in most laboratories that perform standard PCRs, multiple reactions are required. Furthermore, assays would probably require some optimisation for different models of thermal cycler.

#### 1.11.3.3 Hybridisation probe-based assays

The limitations of the original PCR-RFLP assays led to the development of a number of assays that distinguished wild-type from all three mutated sequences by the hybridisation of complementary probes. In most cases, these required multiple reactions with up to four different probes (complementary to the wild type and the three mutated sequences). One hybridisation assay used multiple specific probes that would hybridise and capture complementary amplicon. Subsequent detection of this hybridisation allowed identification of mutation type and so CLA susceptibility (Pina *et al.* 1998). This approach was reported to be accurate when compared with PCR-RFLP and sequencing (Pina *et al.* 1998) and allowed direct testing of gastric biopsies for CLA resistance (Marais *et al.* 1999). A variation of the hybridisation strategy was the development of a homoduplex formation assay, based on the principle that exactly complementary sequences preferentially anneal to form homoduplexes compared with any mismatched sequences. By mixing unknown DNA with double-labelled DNA

duplexes of known sequence, denaturing all DNA and then monitoring the ratio of re-annealed double labelled DNA to single labelled DNA homoduplexes, sequence of the unknown DNA and so CLA susceptibility could be determined (Maeda *et al.* 2000). This approach enabled sensitive detection of CLA susceptibilities directly from gastric aspirates, as well as identifying mixed-susceptibility infections. A different study described an assay that used a panel of fluorescently-labelled probes (wild type plus three probes containing each mutation type) that were hybridised *in situ* to *H. pylori* DNA in gastric biopsies (Trebesius *et al.* 2000). Although this assay was reported to be highly accurate, compared with culture-based methods, and more rapid (3 hr) than PCR, the absence of any amplification step may also decrease the sensitivity of this approach.

Another group described a PCR-line probe assay that amplified 23S rDNA with biotinylated primers. Single-stranded amplicons were subsequently analysed by reverse hybridisation to one of eight different probes immobilised on a nitrocellulose membrane (van Doorn *et al.* 1999a). Unlike other studies, isolates were also examined for the additional mutations G2141A and A2115G that had been proposed to cause CLA resistance in *H. pylori* (Hulten *et al.* 1997) and for mutations A2143C and A2142T, that can be induced *in vitro* (Debets-Ossenkopp *et al.* 1998). However this and a subsequent larger multi-centre study that examined a collective total of 182 CLA-R strains only detected the three commonly described mutations, either singly or in multiple combination. Three strains lacked any of the seven potential mutations (van Doorn *et al.* 1999a;van Doorn *et al.* 2001). This assay was subsequently applied to gastric biopsies (Ryan *et al.* 2001).

#### 1.11.3.4 Real-time PCR probe hybridisation-based assays

Two real-time LightCycler assays that are faster than conventional PCR have been described (Gibson *et al.* 1999;Matsumura *et al.* 2001). In both cases, mutation detection is based on the principle of probe hybridisation melting point analysis, as described in section 2.20.2.2. However while the assay described for the Roche LightCycler required three separate PCR reactions (Matsumura *et al.* 2001), CLA susceptibility could be determined in a single reaction by the assay designed for the Idaho model (Gibson *et al.* 1999). The development of the latter assay for application directly to gastric biopsies is explored in Chapter 5, and the relative merits of the tests available will be discussed more fully in subsequent sections.

### 1.11.4 MTZ resistance

The mean rates of resistance to the nitroimidazole drug MTZ in Europe were reported as 27 % and 33 % by the two European multi-centre antimicrobial surveillance studies described in section 1.11.1 (Glupczynski *et al.* 2001;Megraud *et al.* 1999). However MTZ resistance levels were considerably higher (> 44 %) in countries such as Italy, Austria, Poland and Finland (Glupczynski *et al.* 2001) and can be as high as 90% in developing countries where MTZ is a key therapeutic agent for parasitic infections (Alarcon *et al.* 1999). Culture-based surveillance of MTZ resistance is difficult as there are no standardised methods for susceptibility testing, so inter-laboratory reproducibility and interpretation of results is problematic. This complicates comparison of studies examining effects of MTZ resistance on treatment outcome. However, one meta-analysis of 91 studies that evaluated various triple and quadruple therapy combinations suggested that treatment success decreased from an average of more than 90 % to less than 75 % if the patient was infected with a nitroimidazole-

resistant strain (van der Wouden *et al.* 1999). Similarly a later meta-analysis of a total of 3594 isolates from 65 studies showed an overall risk of treatment failure of 37 % (Dore *et al.* 2000a). Thus, as discussed for CLA, there is a need for enhanced surveillance of MTZ resistance. Problems associated with culture-based susceptibility testing could be overcome by the development of simple molecular tests for MTZ resistance, analogous to those described for CLA in section 1.11.3. However the development of these has not been possible to date, as the understanding of the mechanism of MTZ action and also resistance development in *H. pylori* remains incomplete. An overview of the current hypotheses will follow.

### 1.11.5 MTZ activation and resistance in anaerobic bacteria

MTZ is administered in an inactive form and enters bacterial cells by passive diffusion where it is reduced to form a toxic nitro anion radical (Figure 1.5) as well as nitroso groups and hydroxylamine that damage bacterial DNA, leading to cell death.

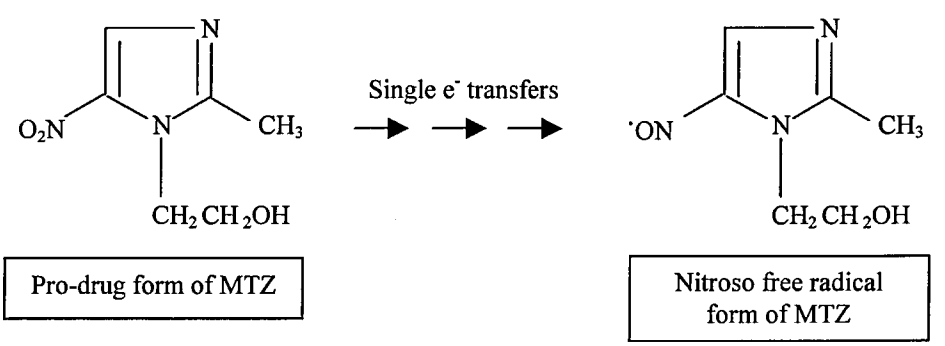



**Figure 1.5 Reduction of MTZ pro-drug by donation of electrons** (Land and Johnson 1999).

As MTZ has a low redox potential, this can only occur in anaerobic or microaerophilic organisms that have pathways with suitably low redox potentials. In

the electron transport chain of anaerobic bacteria, electrons produced by pyruvate oxidoreductase (POR) are transferred via ferredoxin or flavodoxin proteins to a terminal electron acceptor such as a proton. MTZ accepts electrons from these proteins in the cell cytoplasm, creating a concentration gradient that increases the rate of drug uptake (Land and Johnson 1999). Resistance develops by inactivation of components of the electron transport chain, particularly POR and ferredoxin (Jenks and Edwards 2002).

### 1.11.6 MTZ resistance in *H. pylori*

As oxygen tensions are higher in microaerophilic conditions, it was thought that this may allow oxidisation of nitro anion radicals to re-form the MTZ pro-drug as well as toxic oxygen by-products that could damage DNA. However, subsequent investigation of this possibility failed to support the hypothesis of MTZ activity by 'futile cycling' (Smith and Edwards 1995a). Evidence that MTZ was activated by reduction in *H. pylori* rather than futile cycling was provided by early studies, including one that demonstrated increased MTZ uptake in conditions that influence the environmental redox potential and thus rate of MTZ reduction (Smith and Edwards 1995b) and a later study that demonstrated slower MTZ reduction in MTZ-R strains (Smith and Edwards 1997). Some of the bacterial enzymes that may be involved in this reduction process and resistance development are considered in subsequent sections.

#### 1.11.6.1 The potential role of *rdxA* in MTZ resistance of *H. pylori*

An oxygen-insensitive NADPH nitroreductase encoded by the *rdxA* gene has been the focus of numerous investigations following a report that *rdxA* inactivation was associated with the MTZ resistant (MTZ-R) phenotype (Goodwin *et al.* 1998). In the original study, a cosmid library was constructed from a MTZ-R strain and the cosmid

DNA capable of transforming a MTZ-S strain to a MTZ-R phenotype identified. Sequence analysis of this revealed a protein with high-level homology to classical oxygen independent NADPH nitroreductases. Subsequent cloning of *rdx*A from a MTZ-S strain and transformation of this into the intrinsically MTZ resistant *E. coli* resulted in a MTZ-S phenotype and a marked increase in nitroreductase activity. Furthermore, sequencing of *rdxA* in MTZ-S and MTZ-R clinical isolates recovered from patients before and after eradication therapy revealed a range of different genetic mutations in the MTZ-R isolates only. It was proposed that MTZ is activated by RdxA-mediated reduction in sensitive strains and that resistance develops as a result of functional inactivation of this gene (Goodwin *et al.* 1998). Further studies have confirmed that inactivation of the *rdxA* gene can occur by a range of mutation types (Debets-Ossenkopp *et al.* 1999;Jenks *et al.* 1999;Kwon *et al.* 2001a;Paul *et al.* 2001;Solca *et al.* 2000;Tankovic *et al.* 2000). Furthermore mutated *rdxA* genes can transform MTZ-S strains to a resistant phenotype and conversely lethal effects of MTZ can be restored in resistant strains by introduction of functional *rdxA* (Goodwin *et al.* 1998;Jeong *et al.* 2000;Paul *et al.* 2001;Wang *et al.* 2001). Introduction of MTZ resistance by construction of *rdxA* knockout mutants (Kwon *et al.* 2000a;Kwon *et al.* 2000b;Kwon *et al.* 2001b) and virtual absence of *rdxA* mRNA levels (Kwon *et al.* 2000c) and RdxA protein expression (Latham *et al.* 2001) in MTZ-R strains provides further evidence of the importance of *rdxA* in the MTZ resistance mechanism. The contribution of genetic mutations in *rdxA* to MTZ resistance in the UK will be explored in Chapter 10.

#### 1.11.6.2 The potential role of the *frxA* gene in MTZ resistance of *H. pylori*

Although there is substantial evidence in support of a role for the oxygen insensitive nitroreductase RdxA protein, the occurrence of MTZ-R strains that possess an

apparently wild type *rdxA* is well documented (Goodwin *et al.* 1998;Jenks *et al.* 1999;Kwon *et al.* 2001a;Tankovic *et al.* 2000;Wang *et al.* 2001).

Inactivation of the *frxA* gene that encodes NADPH flavin oxidoreductase, was recently shown to increase the Minimum Inhibitory Concentration (MIC) of MTZ-S strains to resistant levels, whilst dual inactivation in combination with *rdxA* resulted in even higher level MICs. In addition inactivated *frxA* genes from clinical isolates could transform *H. pylori* from a MTZ-S to MTZ-R resistotype (Kwon *et al.* 2000a). In contrast, another study conducted at this time demonstrated that *frxA* inactivation alone was insufficient to confer a MTZ-R phenotype, but it would raise MTZ MIC in *rdxA* deficient mutants (Jeong *et al.* 2000). Further investigations suggested that *frxA* inactivation may slow bacterial killing by MTZ but not cause resistance, and that two types of *H. pylori* could be defined; Type I, where resistance can develop by mutation in *rdxA* only and Type II that requires dual mutation of both *rdxA* and *frxA* for a MTZ-R phenotype (Jeong *et al.* 2001). However, while a survey of 12 clinical isolate pairs confirmed that high-level resistance was linked to mutations in both *rdxA* and *frxA* (Kwon *et al.* 2001a), this and one other study that examined clinical isolates demonstrated that intermediate or low level resistance could occur in isolates that contained mutated *frxA* only (Kwon *et al.* 2001a;Marais *et al.* 2003). It is thus evident that the exact contribution of *frxA* inactivation to MTZ resistance remains controversial and is considered further in Chapter 10.

#### 1.11.6.3 The potential role of the *ahpC* gene in MTZ resistance of *H. pylori*

Alkyl hydroperoxide reductases (AhpC), encoded by the *ahpC* gene, reduce harmful alkyl hydroperoxides to their corresponding alcohol and are found in various bacterial species including some helicobacters (Lundstrom *et al.* 2001;Lundstrom and Bolin 2000). A recent study that compared protein profiles of MTZ-S and MTZ-R strains of

*H. pylori* demonstrated higher oxidase and reductase activities in the MTZ-S strains. These activities were largely localised in a protein band comprising several different proteins, including a homologue of subunit of AhpC (Trend *et al.* 2001). In contrast, a proteomic-based study suggested that AhpC expression is upregulated in MTZ-R strains when grown on MTZ-containing agar, and that this was linked to mutations in *rdxA* (McAtee *et al.* 2001). Thus there is conflicting evidence for a potential role of this protein in MTZ resistance, and no study has yet examined the possible involvement of *ahpC* mutations analogous to those described for other genes.

## 1.12 Virulence factors

As discussed in section 1.4, *H. pylori* infection is associated with a broad spectrum of clinical outcomes in different patients. As a chronic pathogen that occupies the gastric niche, *H. pylori* has evolved a variety of adaptive features and virulence factors. Numerous studies have identified potential factors that are involved in survival and orientation in the gastric mucosa, colonisation and adherence to gastric epithelial cells and evasion of the immune response to establish a chronic infection. Additionally, *H. pylori* can cause tissue damage, either directly or as part of a modulation of the host response. It is now evident that the pathogenesis of *H. pylori* infection is complex and multifactorial, also involving host and environmental factors. Table 1.3 provides a list summarising the key bacterial factors that are currently thought to play a role in the virulence of *H. pylori*.

Two virulence factors, vacuolating cytotoxin (VacA) and cytotoxin associated antigen (CagA), encoded by *vacA* and *cagA*, respectively are the focus of further study in Chapters 6 and 7 and so will be considered in more detail in subsequent sections.

**Table 1.3: The putative virulence factors of *H. pylori*.** (Summarised from reviews by (Gerhard *et al.* 2002;Haas *et al.* 2001;Jenks and Kusters 2000;Moran *et al.* 2002;Shimoyama and Crabtree 1998;Suerbaum and Michetti 2002)

| Virulence Factor* | Potential pathogenic role |
|---|---|
| Urease | Survival in gastric environment; entry into mucous layer; colonisation |
| LPS | Molecular mimicry ($Le^x$ , $Le^y$ antigens) → persistence; ?autoimmunity; colonisation |
| Flagella | Entry into mucous layer; flagellins involved in colonisation |
| Phospholipase A | Colonisation |
| Hop proteins (BabA, SabA) | Adhesion by binding fucosylated $Le^b$ blood group antigen (BabA) or sialyl -$Le^x$ glycosphingolipid (SabA) |
| VacA | Forms channel in epithelial membranes, → provides bacteria with nutrients; stimulates inflammatory response; mitochondrial apoptosis; ?decreases gastric mucin levels |
| Cag PAI | Recruitment of inflammatory cells by IL-8 induction; possible host cytoskeletal rearrangements |
| HpNAP | Induces mast cell degranulation and IL-6 release → recruits neutrophils and monocytes; ?iron acquisition |

*Abbreviations are fully defined in the Glossary section.

### 1.12.1 Vacuolating cytotoxin (VacA)

Approximately 50 % of *H. pylori* display vacuolating activity *in vitro* (Leunk *et al.* 1988) due to production of a cytotoxin of approximately 87 kDa (Cover and Blaser 1992), but virtually all strains possess *vacA*, the gene that encodes this protein (Cover 1996). The *vacA* gene, characterised by four independent groups in 1994 (Dunn *et al.*

1997), is 3.9 kb in length and encodes a 139 kDa protoxin, from which a 33-kDa leader sequence is cleaved to yield the mature toxin (Cover *et al.* 1994).

Analysis of Tox + and Tox – strains by PCR and Southern hybridisation demonstrated differences in the middle of the *vacA* sequence (Cover *et al.* 1994). Further alignment of *vacA* middle region sequences from Tox + and Tox – strains demonstrated that these regions were closely related within the two respective phenotypes but were markedly different between Tox + and Tox – strains. Two different families were thus proposed, type m1 alleles, and type m2 alleles that contain an insertion of 23 amino acids (Atherton *et al.* 1995). Further examination of sequences encoding the 33 amino acid signal sequence of VacA identified two basic families of signal sequences, those from tox + strains (allele s1, that could be subdivided further into type s1a and type s1b) and those from tox – strains (allele s2) (Atherton *et al.* 1995). Further examination of the s1 allele demonstrated that sub-type s1a comprised two distinct types, designated s1a and s1c (van Doorn *et al.* 1998b). VacA of s2 strains was subsequently shown to have an additional 12 amino acids that would appear to account for the lack of cytotoxic activity, compared with s1 isolates (Letley and Atherton 2000). Determination of s type, on the basis of amplicon size following PCR, and of m type, by specific probe hybridisation and two PCR assays, in 59 clinical isolates demonstrated that this gene has a mosaic structure, with different allele combinations (s1m1, s1m2 and s2m2) possible (Atherton *et al.* 1995). Genotype s2m1 was subsequently reported but is a rare occurrence (Letley *et al.* 1999). A summary of the structure of the *vacA* gene and encoded polypeptide is presented in Figure 1.6.

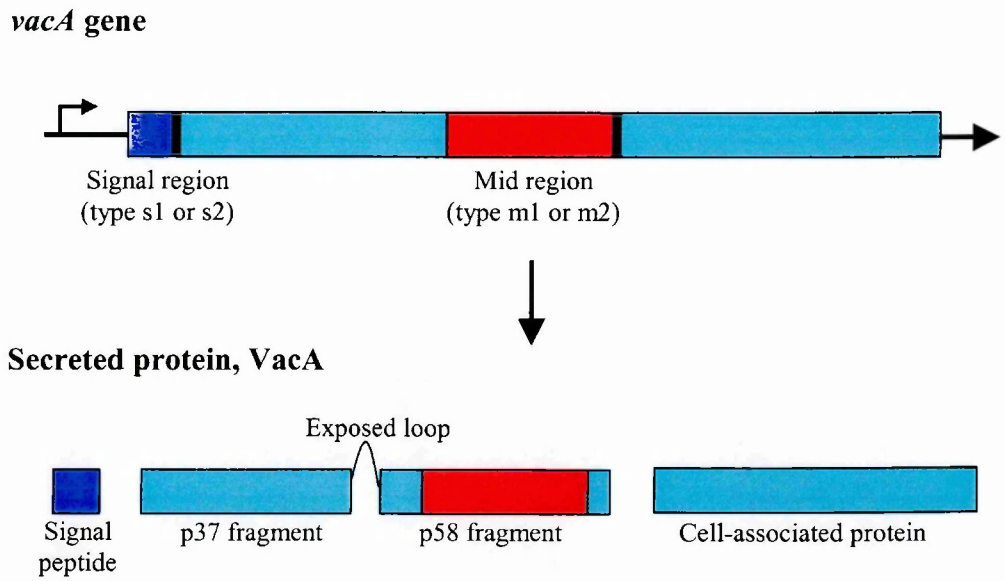



**Figure 1.6 Schematic representation of the vacuolating cytotoxin gene (*vacA*) and protein (VacA), not to scale.** Alleles can be classified according to signal and mid region type. The translated polypeptide is a protoxin, processed during secretion (Atherton *et al.* 2001).

The Atherton study in 1995 suggested that s1m1 isolates were more toxigenic than those of s1m2 and s2m2 genotype, and that the s1 allele was more frequently associated with peptic ulcer disease. Previous studies had suggested that strains associated with peptic ulcer disease were more likely to produce cytotoxin (Tee *et al.* 1995), and it was hypothesised that type s2 isolates export the VacA protoxin less efficiently across the bacterial cytoplasmic membrane, or that differences in signal sequence cleavage sites may alter protein function. Alternatively s types may be markers for other unidentified elements that influence cytotoxic activity (Atherton *et al.* 1995). This early study stimulated numerous investigations of potential associations between *vacA* genotype and virulence potential, described below.

#### 1.12.1.1 Geographical distribution and disease associations of *vacA* genotypes

Sub-types of s1 appear to vary with geographical location. Type s1a predominates in Australia and Northern and Eastern Europe, type s1b is more prevalent in Southern Europe, Africa, Central USA and South America, while most isolates from Eastern Asia are type s1c (Andreson *et al.* 2002;de Gusmao *et al.* 2000;Karhukorpi *et al.* 2000;Kidd *et al.* 1999;Mattar and Laudanna 2000;Strobel *et al.* 1998;van Doorn *et al.* 1998b;van Doorn *et al.* 1998a;van Doorn *et al.* 1998c;van Doorn *et al.* 1999b;Yamaoka *et al.* 1999). However no specific association with disease and s1 sub-type was reported in most of these studies. In contrast, many of these and other studies that analysed either clinical isolates or gastric biopsies directly reported significant associations between the s1 allele and peptic ulcer disease (Arents *et al.* 2001;de Gusmao *et al.* 2000;Evans *et al.* 1998;Kidd *et al.* 1999;Rudi *et al.* 1999;Strobel *et al.* 1998;van Doorn *et al.* 1998a;van Doorn *et al.* 1998c;van Doorn *et al.* 1999b) but other studies did not substantiate this (Andreson *et al.* 2002;Gold *et al.* 2001;Gunn *et al.* 1998;Yamaoka *et al.* 1999). Some evidence has been presented to suggest an association between *vacA* genotype s1m1 and development of gastric cancer also (Evans *et al.* 1998;Kidd *et al.* 1999;Miehlke *et al.* 2000;Miehlke *et al.* 2001). The *vacA* m type has been reported to correlate with *in vitro* toxicity and gastric epithelial damage *in vivo*, but not with peptic ulceration (Atherton *et al.* 1997). These observations may explain the overall lack of apparent association between m type and PUD reported in most of the studies described. Interestingly, however, potential differences in m type of PUD and gastric cancer isolates has been proposed (van Doorn *et al.* 1999b).

It is evident that the association between *vacA* genotype and specific disease remains inconclusive. Further examination of larger study populations from wide

geographical areas may improve understanding of this. However the current tools available for determination of *vacA* genotype, described below, do not appear to be amenable to large-scale application.

### 1.12.1.2 Methods of *vacA* genotyping

Most studies determined *vacA* genotype by multiple PCR assays, not only to distinguish s1 and s2 and m1 and m2 alleles, but also to sub-type s1. Overall the preferred strategy for *vacA* genotyping has been to use the original primers described by Atherton *et al*, (1995) (Evans *et al.* 1998;Gunn *et al.* 1998;Ito *et al.* 1997;Letley *et al.* 1999;Miehlke *et al.* 2000;Miehlke *et al.* 2001;Rudi *et al.* 1999;Strobel *et al.* 1998). Often s type has been defined using additional primers developed to distinguish sub-types s1a and s1c (de Gusmao *et al.* 2000;Karhukorpi *et al.* 2000;Yamaoka *et al.* 1999). However the original primers (VA3-F and VA3-R and VA4-F and VA4-R) could not determine m type in some isolates due to sequence variation at the primer binding sites (Gunn *et al.* 1998;Ito *et al.* 1997;Strobel *et al.* 1998). This led to development of a simpler method that could differentiate types m1 and m2 on the basis of amplicon size with a single PCR reaction using one primer pair (VAG-F and VAG-R) (Atherton *et al.* 1999). Several studies have adopted these primers as a first or second-line strategy for mid region genotyping (Karhukorpi *et al.* 2000;Kidd *et al.* 1999;Yamaoka *et al.* 1999). These PCR assays also allow determination of *vacA* genotype directly from gastric biopsies, with reported sensitivities of > 90 % (Gunn *et al.* 1998;Mattar and Laudanna 2000;Rudi *et al.* 1999).

One alternative strategy was a line probe assay (LiPa) that first involves two separate PCRs to amplify *vacA* signal and mid regions using two and five biotinylated primers, respectively. Labelled amplicons were then added in a reverse hybridisation reaction to a panel of probes, immobilised on nitrocellulose strips, that corresponded

to eight different s types and five m types (van Doorn *et al.* 1998a). Numerous multi-centre studies have applied LiPa to provide more specific and detailed definition of *vacA* genotypes, discriminating within the s and m alleles. Furthermore this approach has demonstrated that mixed genotype infections are not uncommon (Gold *et al.* 2001;van Doorn *et al.* 1998b;van Doorn *et al.* 1999b;van Doorn *et al.* 2000a;van Doorn *et al.* 2000b;van Doorn 2001). The LiPa method has been shown to be a highly sensitive means of *vacA* genotyping directly from gastric biopsies (Arents *et al.* 2001;van Doorn *et al.* 1998c). However, LiPa is a more complex and expensive method than simple PCR and this is likely to limit its use, unless commercially synthesised probe strips become available.

PCR-based *vacA* genotyping has the advantage that it is sensitive, rapid and relatively simple. However the requirement for multiple reactions increases overall workload and cost of these tests. Chapter 6 describes the development and evaluation of a single-step multiplex assay for *vacA* genotyping. One other such assay that is described in the literature (Han *et al.* 1999) will be discussed further in that chapter.

### 1.12.2 Cytotoxin associated gene (CagA)

Early studies reported that culture supernatant of cytotoxic *H. pylori* strains contained a protein ranging from 120 – 130 kDa in size. The purified protein was shown to lack cytotoxic activity but to be associated with the cytotoxin encoded by *vacA* (Cover and Blaser 1992). Subsequent construction and screening of an expression library from a cytotoxic strain demonstrated that the protein was encoded by a large, variable, gene of approximately 3.5 kb, that was named cytotoxin associated gene (*cagA*) (Covacci *et al.* 1993). Western blotting had demonstrated that the immunodominant CagA protein was only expressed in cytotoxic strains, and probe hybridisation analyses suggested that this was due to absence of the *cagA* gene in non-cytotoxic strains

(Covacci *et al.* 1993). Patients with gastroduodenal pathologies have been shown to have significantly higher anti-CagA antibody titres than for patients with normal gastric mucosa (Atherton 1998;Covacci *et al.* 1993). However, while analysis of 43 clinical isolates demonstrated that most, but not all, strains could be subdivided into type I strains that possess *cagA* and express VacA protein and type II strains that lack *cagA* and are non-cytotoxic, strong associations between type I strains and duodenal ulceration could not be found (Xiang *et al.* 1995). Although inactivation of *cagA* does not affect VacA expression or the ability of a strain to induce the pro-inflammatory cytokine interleukin 8 (IL-8) (Tummuru *et al.* 1994), genomic mapping demonstrated that *cagA* was part of a 40-kb region of about 30 genes that had a different G + C content from the rest of the *H. pylori* genome (Censini *et al.* 1996). This region had features analogous to those of bacterial pathogenicity islands; fragments of DNA acquired later in bacterial evolution that enhance strain virulence, and so was named the *cag* pathogenicity island (*cag* PAI) (Akopyants *et al.* 1998;Censini *et al.* 1996).

The *cag* PAI contains genes homologous with a type IV secretion system that may be involved in export of CagA or other proteins (Akopyants *et al.* 1998;Censini *et al.* 1996;Odenbreit *et al.* 2000). Additionally inactivation of several individual genes of the PAI, excluding *cagA*, can abolish the ability of a strain to induce IL-8 *in vitro* (Akopyants *et al.* 1998;Censini *et al.* 1996;Tummuru *et al.* 1995). The structure of the *cag* PAI can vary, being either continuous or split in two (Akopyants *et al.* 1998;Audibert *et al.* 2001;Censini *et al.* 1996), as summarised in Figure 1.7. While both structures can induce IL-8, it is interesting to note that some strains that lack an intact *cag* PAI can also induce IL-8, albeit rarely, indicating the involvement of other bacterial factors in this process (Audibert *et al.* 2001). Furthermore, while a high proportion of strains associated with duodenal ulceration contain the *cag* PAI, strains

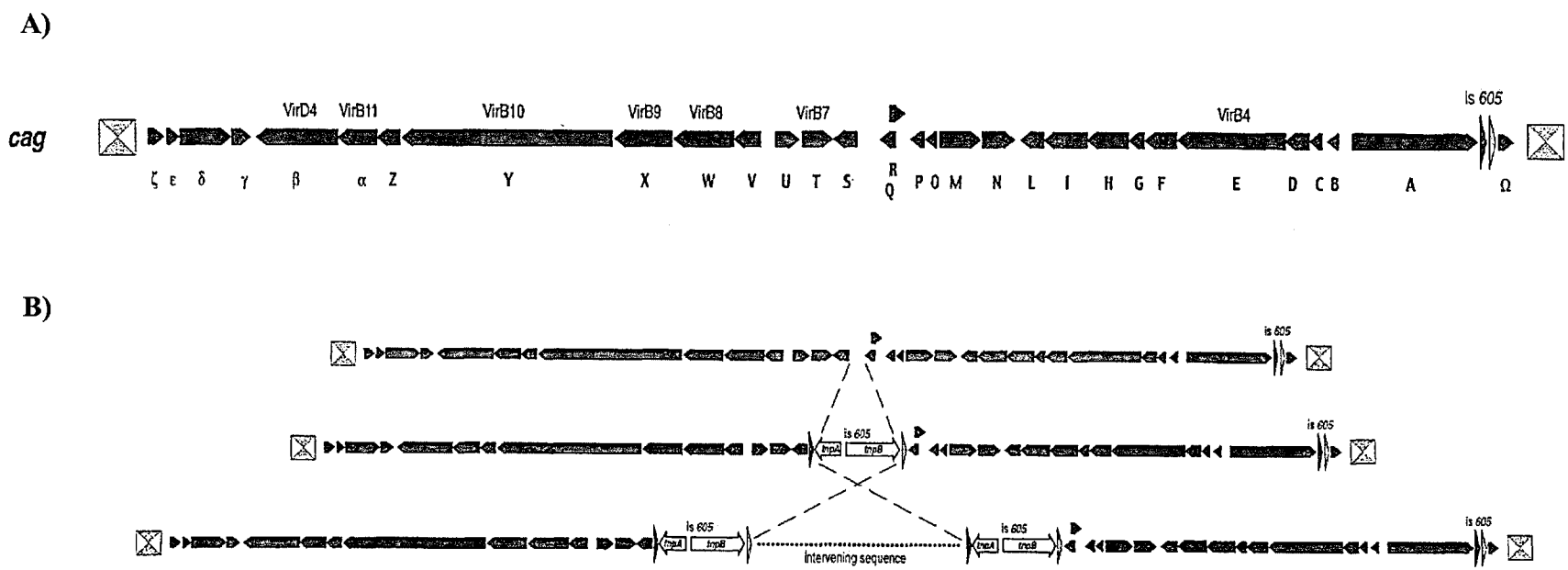



**Figure 1.7 The organisation of *cag* pathogenicity island genes. (Reproduced from Stein *et al.* 2001)**

**A)** The open reading frames within the 40-kb *cag* PAI are depicted as arrows with the *cag* gene designations (single letters) below and names of homologues above. **B)** Three alternative arrangements of the *cag* PAI from three additional *H. pylori* strains. IS *605* is an insertion sequence, *tnpA* and *tnpB* are transposons A and B.

lacking this can also be found in DU patients (Audibert *et al.* 2001;Jenks *et al.* 1998) The precise link between *cagA,* the production of the CagA protein, and strain virulence thus remains controversial (Graham and Yamaoka 2000;Mitchell *et al.* 1996;Peters *et al.* 2001). About 68 % of strains isolated from dyspeptics in the United Kingdom have *cagA* present in the genome (Owen *et al.* 2001) and similar or higher frequencies have been reported in France (Jenks *et al.* 1998), Japan (Maeda *et al.* 1998a) and Costa Rica (Occhialini *et al.* 2001). Nevertheless, *cagA* status alone is insufficient to reliably predict either the virulence of *H. pylori* or its association with gastric ulcer (Blaser and Berg 2001).

Until recently, the biological role of the CagA protein was not known. However there is now considerable evidence to suggest that CagA is involved in inducing cytoskeletal rearrangements in epithelial cells, as summarised in Figure 1.8. Investigation of the interaction of epithelial cells with wild type *H. pylori* and mutant strains lacking components of the *cag*PAI demonstrated that, following attachment of *cagA*-positive *H. pylori* to host epithelial cells, CagA is exported from the bacterial cell via the type IV secretion system encoded by the *cag*PAI, and translocated into epithelial cells (Odenbreit *et al.* 2000;Stein *et al.* 2000). An earlier study had demonstrated that infection of epithelial cell lines with *H. pylori* led to phosphorylation of an unknown protein of 145 kDa and pedestal formation (Segal *et al.* 1996), later defined as the "hummingbird phenotype" (Segal *et al.* 1999). Several groups subsequently identified the unknown protein as CagA, that, following translocation, localises in the epithelial membrane in proximity to the site of bacterial adhesion (Asahi *et al.* 2000;Backert *et al.* 2000;Odenbreit *et al.* 2000;Segal *et al.* 1999;Stein *et al.* 2000). Tyrosine residues of CagA are phosphorylated by host cell kinases (Asahi *et al.* 2000;Backert *et al.* 2000;Odenbreit *et al.* 2000;Odenbreit *et al.*

2001;Segal *et al.* 1999;Stein *et al.* 2000) including those of the c-Src and Lyn families (Stein *et al.* 2002) and this in turn activates eukaryotic signal transduction pathways and cytoskeletal plasticity. A recent study of a cell line (AGS) transfected with CagA demonstrated that $CagA^{TyrP}$ interacts with the SH 2 domain of SHP-2, a cytoplasmic tyrosine phospatase, leading to upregulation of phosphatase activity that is central to development of the hummingbird phenotype, either directly or by dephosphorylating other host cell proteins (Higashi *et al.* 2002). It is proposed that the resultant abnormalities in gastric epithelial cell proliferation and movement may play a critical role in the development of disease, particularly gastric cancer. The recent demonstration of $CagA^{TyrP}$-SHP-2 complexes, only in the gastric mucosa of patients with atrophic gastritis may indicate the involvement of this process in early stage carcinogenesis (Yamazaki *et al.* 2003).

Three putative tyrosine phosphorylation motifs in the CagA protein were predicted with the MOTIF algorithm and designated TPM-A (KFGDQRY), TPM-B (KNSTEPIY) and TPM-C (KLKDSTKY) (Odenbreit *et al.* 2000;Odenbreit *et al.* 2001) although other analyses predict additional motifs (Evans, Jr. and Evans 2001) such as the EPIYA sequences in the repeat region of CagA (Stein *et al.* 2000). The relationship between *cagA* TPM and clinical progression of *H. pylori* infection is not well defined. To date, most studies have characterised TPM status of a strain by sequencing regions of *cagA*. While this approach allows accurate identification of TPMs, the high associated reagent and labour costs limits the number of isolates that can be surveyed and prevents extensive evaluation of the clinical significance of *cagA* TPMs. Chapter 7 describes the development and application of three novel real-time LightCycler assays for rapid screening of a larger study population to examine potential associations of specific TPMs with disease.

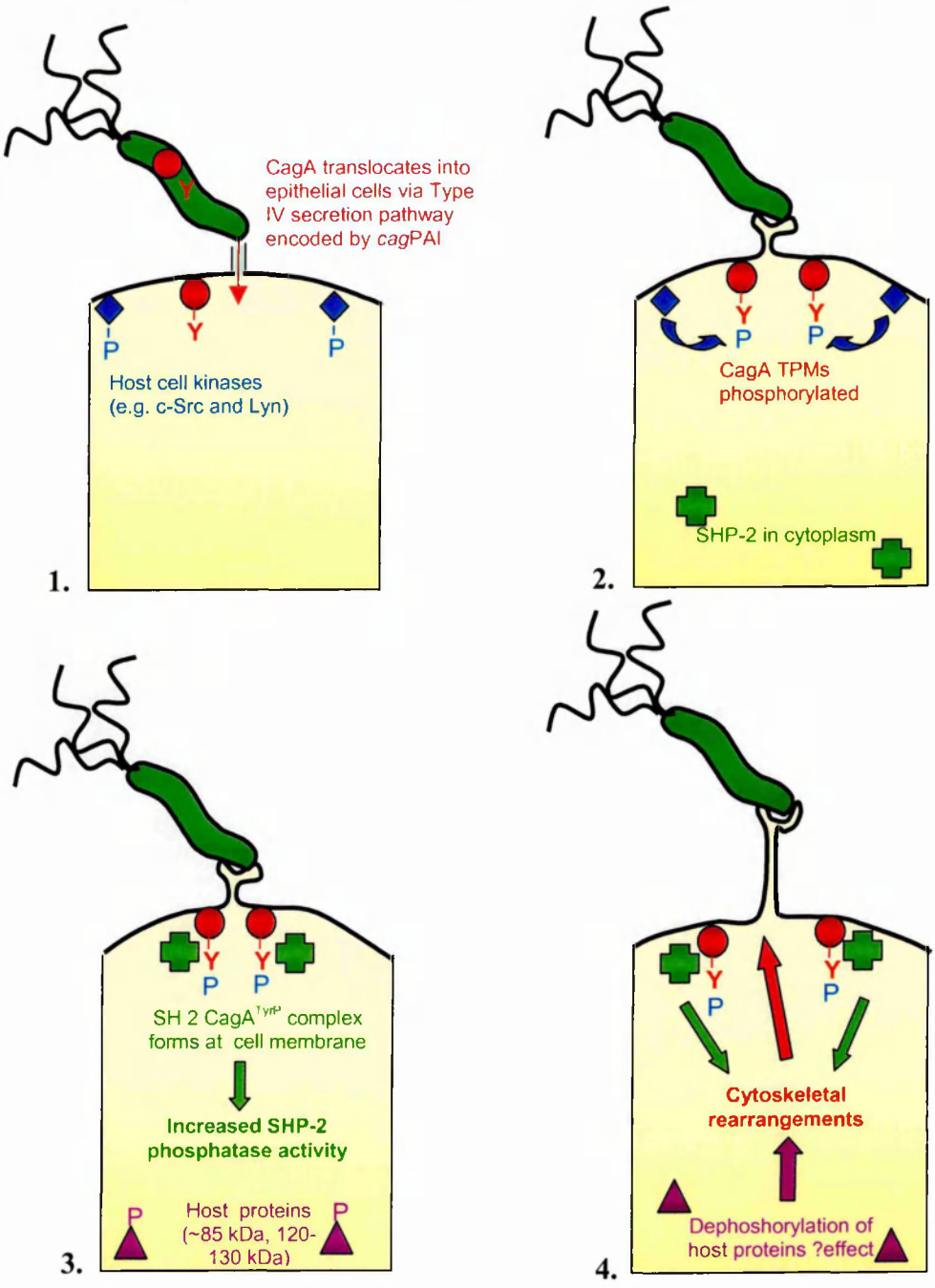



**Figure 1.8** **Schematic representation of the interactions between *H. pylori* CagA and host epithelial cells.** (Compiled from Segal *et al.* 1999;Stein *et al.* 2002)). *H. pylori* binds to epithelial cells and translocates CagA (**1**) that is then phosphorylated on TPMs by host kinases (**2**) leading to upregulation of host phosphatases (**3**), protein dephosphorylation and host cytoskeletal rearrangements (**4**).

## 1.13 Other helicobacters associated with human gastric infection

Although *H. pylori* is the predominant human gastropathogen, gastric infection with '*H. heilmannii*' (Hilzenrat *et al.* 1995;Holck *et al.* 1997;Ierardi *et al.* 2001;Jalava *et al.* 2001;Jhala *et al.* 1999;McNulty *et al.* 1989) and, rarely, *H. bizzozeronii* (Jalava *et al.* 2001), *H. felis* (Germani *et al.* 1997;Lavelle *et al.* 1994) and *H. cinaedi* (Pena *et al.* 2002) have been reported also, and are considered further in subsequent sections.

### 1.13.1 History of '*H. heilmannii*'

Spiral organisms that were morphologically distinct from *H. pylori* had been reported in the stomach of various animals , but the only early description of such bacteria in the human stomach was by Krienitz in 1906 (Kidd and Modlin 1998). It was not until the discovery of *H. pylori* almost 80 years later, and the consequent intensive investigation of the human gastric mucosa, that these organisms were reported in three patients (Dent *et al.* 1987). In 1989, spiral bacteria were observed by Gram stain, histology or by electron microscopy in the gastric mucosa of six patients, all of whom had histological evidence of gastritis, similar to that induced by *H. pylori.* The distribution of the organism was patchy and sparse and it could not be cultivated under conditions that promotes growth of *H. pylori.* However, there were sufficient numbers of organisms present in one biopsy to produce a positive result by RUT. Repeat testing of patients over a number of months demonstrated that, like *H. pylori*, this was a chronic infection. It was proposed that the organism be classified on the basis of its morphology along with other similar gastric spiral bacteria, into a new genus *Gastrospirillum*, and the organism observed in these patients was named *G. hominis* (McNulty *et al.* 1989). Subsequent analyses of urease genes as well as 16S rDNA demonstrated that this organism was likely to be part of the *Helicobacter* genus

(Solnick *et al.* 1993;Solnick *et al.* 1994) and *G. hominis* was renamed *'Helicobacter heilmannii'*, although this has not yet been formally validated.

'*H. heilmannii*' infection has since been shown to have a high incidence (78 – 100 %) in a wide range of other animal hosts including cats, dogs and pigs (De Groote *et al.* 1999;Eaton *et al.* 1996;Neiger *et al.* 1998).

### 1.13.2 Definition of "*H. heilmannii*-like" organisms (HHLO)

Although the nomenclature implies a defined species, 16S rDNA phylogenetic analyses demonstrate that *'H. heilmannii'* comprises two related but distinct types (O'Rourke *et al.* 2001;Solnick *et al.* 1993). Type 1 is closely related to '*Candidatus* H. suis', found in pigs (De Groote *et al.* 1999), whereas Type 2 is more closely related to the *H. bizzozeronii/H. felis/H. salomonis* group that most commonly infect cats and dogs (Jalava *et al.* 1997;Solnick *et al.* 1993). These morphologically complex, often non-culturable bacteria, from various human and animal hosts represent part of an ill-defined taxonomic group of closely related forms which, for convenience, have been referred to as '*Helicobacter heilmannii*'-like organisms (HHLO).

### 1.13.3 Clinical features, incidence and diagnosis of human HHLO infection

Human HHLO infection possibly occurs via a zoonotic route, and may lead to the development of PUD (Hilzenrat *et al.* 1995;Jhala *et al.* 1999) or low-grade MALT lymphoma (Morgner *et al.* 2000).

The reported incidence of human '*H. heilmannii*' infection based on histology is low (0.1 - 2 %) in dyspeptic adults (Ierardi *et al.* 2001;Jhala *et al.* 1999;Svec *et al.* 2000) and in paediatric patients (Mention *et al.* 1999;Svec *et al.* 2000). As HHLOs from human tissue remain uncultivable with rare exceptions (Andersen *et al.* 1996), diagnosis is primarily reliant on histology, although touch cytology is also described (Debongnie *et al.* 1994). Recently, another microscopy-based system, fluorescent *in*

*situ* hybridisation (FISH), was described that detected, by a panel of probes, all known types of '*H. heilmannii*' and several new forms direct from paraffin embedded gastric biopsies (Trebesius *et al.* 2001). PCR-based studies have identified HHLOs in both human (Morgner *et al.* 2000) and animal (Norris *et al.* 1999) gastric biopsies by amplification of *Helicobacter* genus-specific fragments of 16S rDNA followed by sequencing. One PCR detection assay, based on the *ureB* gene was described for detection of '*H. heilmannii*' in domestic cat biopsies (Neiger *et al.* 1998), but specific PCR has only been applied for detection of human infection in a single patient (Dieterich *et al.* 1998). A number of histologically-based studies have provided valuable information on the clinical features of HHLO infections (Holck *et al.* 1997;Jhala *et al.* 1999;Mention *et al.* 1999;Stolte *et al.* 1994) and have suggested that when compared with *H. pylori*, HHLO distribution tends to be focused in the antrum (Jhala *et al.* 1999) as well as being patchy and sparse (Holck *et al.* 1997). Microscopy-based methods of detection can be relatively insensitive when bacterial numbers are low (Holck *et al.* 1997;Megraud 1995a). In the original case report of this infection, it was stated that slides had to be examined for at least five minutes, to minimise falsely negative results (McNulty *et al.* 1989). It is thus possible that the incidence of human HHLO infection may be under-reported. Development and evaluation of a novel PCR-based method to detect HHLOs is presented in Chapter 4.

### 1.14 Helicobacters associated with human extra-gastric infection

Several helicobacters that typically occupy extra-gastric niches in a range of non-human animal reservoirs have been cultured from human diarrhoea specimens (Table 1.1). *H. cinaedi* and *H. fennelliae* have both been associated with chronic enteritis and proctitis (Totten *et al.* 1985), in immunocompromised patients and less frequently in immunocompetent individuals (Fox 2002). *H. cinaedi* also has the potential to

become bacteraemic and cause systemic disease (Hung *et al.* 1997;Kiehlbauch *et al.* 1994;Orlicek *et al.* 1993;Tee *et al.* 1996). Several reports suggest potential associations between human *H. pullorum* infection, probably acquired from chickens, and development of gastroenteritis (Burnens *et al.* 1994;Stanley *et al.* 1994;Steinbrueckner *et al.* 1997). Other species recovered from human diarrhoeal specimens include *H. canis*, *H. canadensis*, *H. winghamensis*, '*H. rappini*' and '*H. westmeadii*' (Fox 2002), although the causal relationship between these infections and gastroenteritis is less well-understood (O'Rourke *et al.* 2001). As has been demonstrated in section 1.9, numerous studies have presented PCR-based evidence of extra-gastric *Helicobacter* infections also and this is explored further in Chapter 9.

## 1.15 Aims and Objectives

This study was initiated as part of a PHLS funded research project that aimed to explore and develop the role of conventional and real-time PCR in the investigation of human *Helicobacter* infections. Within this aim are several specific objectives.

1. To examine the factors that could influence outcome of PCR-based analyses of clinical specimens, including sample transport conditions, DNA extraction methods and primers used.
2. To develop and evaluate PCR assays to detect all species of *Helicobacter* as well as specifically *H. pylori* and '*H. heilmannii*'-like organisms, and to apply these in the examination of gastric and extra-gastric chronic inflammatory disease.
3. To explore non-invasive detection of *H. pylori* in stool specimens by PCR compared with specific antigen detection.
4. To apply PCR-based methodologies in the examination of antibiotic resistance in *H. pylori*, for CLA susceptibility testing direct from gastric biopsy and for further exploration of the mechanism of MTZ resistance.
5. To develop and evaluate PCR based assays for the investigation of putative virulence factors *vacA* and *cagA*, both from culture and direct from gastric biopsy.

A PCR-based approach not only has the facility to allow rapid, potentially non-invasive, detection of helicobacters associated with human disease, but could provide further strain information that could directly impact on patient management as well as enhancing surveillance of specific markers of antibiotic resistance and strain virulence.

# Chapter 2. MATERIALS AND METHODS

Unless stated otherwise, all reagents and reactions were prepared or performed in sterile distilled water. Latex gloves were worn for all molecular methods and for all procedures handling clinical specimens. All routine culture media used were prepared in-house by the CPHL media preparation suite. Manufacturers and suppliers are given in squared parentheses. Reference strains are listed in Appendix A.

## 2.1 Bacterial isolates used

### 2.1.1 Sources of bacterial isolates

Type strains were obtained from the National Collection of Type Cultures (NCTC) in lyophilised form (Appendix A). Other bacterial isolates were obtained from the stored (-80 °C) Helicobacter Reference Unit (HRU) collection, comprising strains referred routinely from primary diagnostic laboratories that cultured *H. pylori* from gastric biopsy and also from collaborating laboratories world wide. The collection also contained isolates cultured in HRU from gastric biopsies referred routinely from collaborating gastroenterology clinics and microbiology laboratories. Exact numbers of isolates used are provided in subsequent chapters.

### 2.1.2 Culture and storage of bacterial isolates

All bacterial strains were cultured on Columbia Blood agar (CBA) - Columbia agar base CM331 [Oxoid Ltd, Basingstoke, Hampshire, UK] with 10 % (*v/v*) defibrinated horse blood [E&O Laboratories, Bonnybridge, UK] - at 36 °C in a variable atmospheric incubator (VAIN) [Don Whitley Scientific Ltd, Shipley, West Yorkshire, UK] which maintained a microaerophilic atmosphere of 4 % $O_2$, 5 % $CO_2$, 86 % $N_2$, 5

% $H_2$. Stock cultures were stored long-term in duplicate by preparation of a heavy bacterial suspension in a sterile vial containing commercial cryopreservative and 25 porous glass beads [Microbank™, Pro-Lab Diagnostics, Wirral, UK]. Bacterial suspensions were mixed with the beads by gentle inversion. Excess liquid was transferred to a second 2-ml screw-cap tube containing 20-30 glass beads, mixed and liquid discarded. Beads were stored (–80 °C) in two separate freezers.

### 2.1.3 Recovery of stored cultures

Beads were streak-plated onto CBA with a plastic sterile 10 µl loop [Technical Services Consultant, Heywood, Lanes, UK] and incubated at 36 °C under microaerobic conditions for 48 - 72 h and then sub-cultured onto CBA and incubated for 48 h as described in section 2.1.2.

## 2.2 Transport of cultures and biopsies

Clinical specimens can contain contaminating bacteria that multiply rapidly, suppressing growth of slow-growing organisms like *H. pylori*. Inclusion of antibiotics in transport media can partially overcome this. Vancomycin inhibits growth of most Gram-positive bacteria; trimethoprim lactate and cefsulodin suppress growth of other Gram-negative bacteria, while amphotericin B inhibits fungal growth.

All biopsies referred to HRU for culture or molecular analyses and all referred cultures were transported in Dent's transport medium - 3.7 % (*w/v*) Brain Heart Infusion broth [CM225, Oxoid Ltd], 2.5 % (*w/v*) yeast extract [L21, Oxoid Ltd], 5.0 % (*v/v*) sterile horse serum and *Helicobacter* Supplement [SR147, Oxoid Ltd] (10 µg/ml vancomycin, 5 µg/ml trimethoprim lactate, 5 µg/ml cefsulodin, 5 µg/ml amphotericin B).

Where possible, cultures and biopsies were transported to the HRU with minimal delay (preferably within 24 h). For biopsies that were to undergo molecular analyses and where delays in transport were unavoidable, these were stored at –20 °C and transported on ice to the HRU. The effects of a range of different transport conditions for gastric biopsies on PCR was considered in Chapter 3.

## 2.3 Clinical Specimens

A range of different clinical specimens were examined during the course of this study. Human and feline gastric biopsies (sections 2.3.1 and 2.3.6) were collected from subjects as part of their routine investigation. Other biopsies (sections 2.3.3 – 2.3.5) were collected as part of specific studies investigating clinical conditions for which no microbiological cause has yet been established. The design of these studies in terms of patient inclusion criteria and definition of control subjects was established at the primary participating centre. Molecular analyses were performed (described in Chapter 9) as part of an HRU collaboration with these centres. Where appropriate, ethical approval and patient consent for investigations was obtained locally at each primary clinical centre.

### 2.3.1 Human gastric biopsies

These were collected from patients at endoscopy for diagnostic tests as part of the gastroenterologist's routine investigation of dyspepsia. A total of 317 gastric biopsies included in this work were collected from the hospitals and clinics listed in sections below. The methods by which *H. pylori* status had been determined prior to molecular testing varied according to the local policy for preferred diagnostic tests.

*2.3.1.1 Broomfield Hospital, Chelmsford.* Separate biopsies were collected for histological examination and for culture. Biopsies that had been cultured by the

Chelmsford Public Health Laboratory were sent to the HRU for molecular analyses. All matched *H. pylori* cultures and histology reports were forwarded to the HRU also.

*2.3.1.2 North Middlesex University Hospital, London.* Gastric biopsies were collected and *H. pylori* status determined by rapid urease testing (CLO test [Delta West Pty Ltd, Bentley, Western Australia]) in the department of gastroenterology. CLO test biopsies were then sent to the hospital's PHLS Collaborating Centre where they were batched and sent weekly to the HRU for culture and molecular analyses.

*2.3.1.3 St George's Hospital, London; University College Hospitals, London; Royal Free Hospital, London, King's College Hospital, London.* Gastric biopsies were collected and sent to local microbiology departments from where they were immediately referred to the HRU for culture (section 2.5) and molecular analyses.

### 2.3.2 Stool specimens

Stool samples were provided by dyspeptic patients attending the open access endoscopy clinic at Broomfield Hospital (Chelmsford), collected as part of their routine investigation. All patients who provided stool specimens had given informed consent prior to endoscopy. Stools were brought to the endoscopy clinic in a sterile faecal sample container [Bibby Sterilin Ltd, Stone, Staffordshire, UK] and sent, along with the gastric biopsy taken at endoscopy, to Chelmsford Public Health Laboratory. Stools were then immediately stored at –20 °C, and subsequently transported frozen in batches to the HRU for stool antigen testing (section 2.23) and for molecular analyses.

### 2.3.3 Bladder biopsies

Bladder biopsies were collected from 27 patients with a range of urological symptoms, including Interstitial Cystitis (IC) (n = 10), haematuria and incontinence

(n=17) who were attending a urology clinic for cystoscopy as part of their routine investigation at Broomfield Hospital (Chelmsford).

Biopsies obtained from the left and right bladder wall were, along with urine samples for each patient, cultured for helicobacters at Chelmsford Public Health Laboratory. Biopsies were stored at –20 °C and sent in batches at <0 °C to the HRU for DNA extraction (section 2.11.2) and for further molecular testing.

### 2.3.4 Colonic biopsies

Colonic biopsies were collected from inflamed and non-inflamed areas of the colon from 30 patients undergoing investigation at the Department of Gastroenterology, St Mark's Hospital, Northwick Park, Harrow (Middlesex) and transported on ice to the HRU the same day as colonoscopy. Patient groups had been defined on the basis of their disease state, namely ulcerative colitis (UC) patients (n = 11), Crohn's disease (CD) patients (n = 9) or control patients (n = 10) who had other conditions including rectal bleeding, diarrhoea, polyps and constipation. Local ethical approval had been obtained for this study and all patients gave their informed consent. DNA extraction was performed as described in section 2.11.2 on the day of biopsy receipt. Separate biopsies were examined by routine haematoxylin and eosin staining by histopathologists at St Mark's Hospital.

### 2.3.5 Bronchial biopsies

Bronchial biopsies were collected from 10 patients, all of whom had chronic persistent cough (CPC) and were undergoing bronchoscopy at Taunton and Somerset Hospital (Somerset). PHLS ethical approval had been granted for this study and all participating patients gave informed consent to further investigation for helicobacters.

Biopsies were stored (-20°C) at Taunton PHL and forwarded to the HRU at 4°C. DNA was extracted from biopsies (section 2.11.3) on the day of receipt.

### 2.3.6 Feline gastric biopsies

Gastric biopsies from 15 domestic cats under investigation at The Village Veterinary Practice (Belsize Park, London) for various symptoms were collected and cultured for species of *Helicobacter* as described in section 2.5. Biopsies were examined microscopically by Gram stain (section 2.6.1), and then stored (-20 °C) until DNA was extracted as stated in section 2.11.2.

## 2.4 Faecal spiking experiments

These were conducted following a modification of the protocol described by Lawson *et al* (1997). Bacterial isolates were cultured as described in section 2.1.2. and harvested during the exponential phase of growth (after 48 h). Bacterial suspensions of a turbidity equivalent to a MacFarland's Standard of 1.0 [Pro Lab Diagnostics, South Wirral, Cheshire, UK] were prepared in brucella broth (BB) – 2.0 % (*w/v*) peptone 180, 0.1 % (*w/v*) dextrose, 0.2 % (*w/v*) yeast extract, 0.5 % (*w/v*) sodium chloride, 0.01 % (*w/v*) sodium bisulphite [Invitrogen, Paisley, Strathclyde, UK] - and serially diluted from $10^{-1}$ to $10^{-7}$. Faecal specimens (1 g) were suspended in 9 ml of inoculated Brucella Broth. In subsequent experiments, serial dilutions containing skimmed milk powder [Marvel, Premier Foods, Merseyside, UK] (330 mg/ml), to block binding of PCR inhibitors to silica or diatoms, were emulsified with 1 g stool.

Bacterial cell suspensions were quantified by a viable count method where 20 µl volumes of the $10^{-3}$, $10^{-4}$ and $10^{-5}$ solutions were cultured in triplicate under microaerobic conditions described in section 2.1.2.

## 2.5 Culture of *H. pylori* from gastric biopsies

*H. pylori* was cultured from gastric biopsies by streak-plating with a 10 µl plastic sterile loop [Technical Service Consultants Ltd] on CBA and on *H. pylori* selective Dent's agar - Columbia base [Oxoid Ltd], 7 % (*v/v*) laked horse blood and *Helicobacter* selective supplement [SR1, Oxoid Ltd] - and incubated at 36 °C under microaerophilic conditions. Plates were examined for growth after three, five and seven days.

## 2.6 Characterisation of *H. pylori*

*H. pylori* was identified on the basis of the following simple biochemical tests:

### 2.6.1 Gram stain

The structure of a bacterial cell wall is a key feature in its classification. Gram staining allows cell walls composed of a single membrane and thick peptidoglycan layer (Gram positive) to be distinguished from those that have both inner and outer membranes and less peptidoglycan (Gram negative). The thick peptidoglycan layer of Gram-positive bacteria enables the cell to retain the crystal violet-iodine complex and stain violet. In contrast, this complex is decolourised by acetone in Gram-negative bacteria and cells stain the pale pink colour of the counterstain. *H. pylori* cells typically stain as Gram negative, slender spiral curved rods.

Bacterial cells were emulsified in sterile water on a glass microscope slide [Chance Propper Ltd, Smethwick, Warley, UK] with a sterile loop, air dried and fixed to the glass surface by brief heating. Cells were serially stained with crystal violet [Pro-Lab Diagnostics] for 1 min, Lugol's iodine [Pro-Lab Diagnostics] for 1 min, decolourised by the addition of acetone [BDH, Merck Ltd, Lutterworth, Leicestershire, UK] for 30

s, washed with water, and counter-stained with safranin [Pro-Lab Diagnostics]. Slides were examined under oil immersion at a X 100 magnification.

### 2.6.2 Urease test

*H. pylori* possesses very potent, rapid urease activity and this is a key biochemical test in identification. Urea is catabolised to carbon dioxide and ammonia, resulting in an increased pH that can be detected by a suitable indicator (e.g. phenol red).

Christensen's urea agar slopes - 2.4 % (*w/v*) urea agar base [Oxoid Ltd] 2 % (*w/v*) urea solution [Sigma Ltd, Dorset, UK] - were stab-inoculated with bacterial cells on a sterile loop and incubated at room temperature. A urease reaction was considered positive if the pH indicator changed the colour of the medium from orange to pink within minutes.

### 2.6.3 Catalase test

Many bacterial species, including *H. pylori*, possess the enzyme catalase to survive the toxic by-products of oxygen metabolism. Two molecules of hydrogen peroxide ($H_2O_2$) are hydrolysed to produce an $O_2$ molecule and two $H_2O$ molecules.

Bacterial cells were spotted via a sterile loop into a sterile petri dish [Bibby Sterilin Ltd] and a drop of 3.0 % (*v/v*) hydrogen peroxide [BDH] added. Catalase activity is demonstrated by rapid evolution of bubbles of oxygen gas within 5 s.

### 2.6.4 Oxidase test

Cytochrome oxidase is a vital component of the cytochrome metabolic pathway of many bacterial species, including *H. pylori*, and will oxidise tetramethyl-p-phenylenediamine dihydrochloride in a test strip to indophenol blue.

Bacterial cells were smeared via a sterile plastic loop across the surface of an oxidase test strip [Pyo-test, Medical Wire and Equipment Co Ltd, Wiltshire, UK]. A

positive reaction was recorded if the paper changed colour from white to purple within 5 s.

## 2.7 Antibiotic susceptibility testing

Bacterial susceptibility to antibiotics was assessed *in vitro* by diffusion-based methods - disk diffusion and Epsilometer (E)-test. In both tests a bacterial lawn was applied to the surface of an appropriate agar medium and a disk or strip containing antibiotic was added. The antibiotic diffuses into the media, creating a concentration gradient; the size of which depends on the solubility and molecular weight of the antibiotic as well as the depth of the agar. Plates were incubated for a specified period of time and then examined for bacterial growth. A sensitive organism cannot grow in the presence of antibiotic and a zone of inhibition where the antibiotic has diffused will be observed. In contrast, a resistant organism will grow in the presence of the antibiotic: thus a reduced or no zone of growth inhibition will be seen. Disk diffusion testing provides a qualitative assessment of antibiotic resistance. E-test strips contain a predefined and continuous antibiotic concentration gradient, allowing determination of the minimum concentration at which bacterial growth is inhibited. This minimum inhibitory concentration (MIC) provides a quantitative assessment of the level of bacterial resistance.

### 2.7.1 Disk diffusion method

Approximately $10^7$ cfu (MacFarland's standard 4.0) *H. pylori* cells were suspended in Maximum Recovery Diluent (MRD) - 0.1 % (*w/v*) peptone and 0.85 % (*w/v*) sodium chloride [BDH], pH 7.0 - and a bacterial lawn was prepared by application of this with a sterile cotton tip [Technical Service Consultants Ltd] to CBA using a rotary

plater [Denley, Life Sciences International UK Ltd, UK]. Paper disks impregnated with either clarithromycin (CLA) (2 μg) or metronidazole (MTZ) (5 μg) [Oxoid Ltd] were applied to the surface of the plate. Antibiotic susceptibilities were recorded by measuring the diameter of the zone of inhibition after microaerobic incubation for 48 h at 36 °C. A strain was considered CLA resistant (R) if there was no zone of inhibition, while MTZ resistance was defined as a zone of inhibition ≤ 20 mm.

### 2.7.2 Minimum Inhibitory Concentration (MIC) determination

Bacterial lawns were prepared as described (2.7.1) and E-test strips [AB Biodisk, Solna, Sweden] containing either CLA or MTZ of concentration ranges 0.016 – 256 μg/ml for each were applied to the plate and read after microaerobic incubation for 48 h at 36 °C. MICs were defined as the concentration point at which the zone of inhibition intercepted the E-test strip. CLA resistance was defined as a bacterial strain with an MIC of ≥2 μg/ml. MTZ susceptibilities of bacterial strains were classified on the basis of MIC as sensitive (<8.0 μg/ml) or resistant (≥8.0 μg/ml) (Megraud *et al.* 1999).

## 2.8 Separation of mixed strain populations

All mixed strain populations of MTZ sensitive (S) and resistant (R) phenotypes that were identified by the E-test method were separated by selective and replicate plating.

### 2.8.1 Preparation of MTZ Blood Agar

MTZ stock solutions (16 mg/ml) were prepared by dissolving 0.16 g powdered MTZ [Sigma Ltd] in 0.5 ml dimethyl formamide [Sigma Ltd] and 9.5 ml sterile water. This was then filter-sterilised [0.2 μm MiniSart NML, Sartorius, Göttingen, Germany].

10 % (*v/v*) CBA was prepared by adding 100 ml sterile defibrinated horse blood [E&O Laboratories] to 900 ml Columbia agar [Oxoid Ltd] that had been melted (100 °C for 30 min) and cooled to 50 °C. MTZ CBA plates (8.0 μg/ml) were prepared by the addition of 0.5 ml stock MTZ solution. Following gentle mixing, 20-ml aliquots of media were poured into sterile Petri dishes [Bibby Sterilin Ltd] and once set, stored at 4 °C until used (within 24 h).

### 2.8.2 Separation of mixed MTZ sensitive and resistant populations

MTZ-R sub-populations were separated from MTZ-S sub-populations by subculture on MTZ CBA plates (8 μg/ml) under standard microaerobic growth conditions. To obtain a pure culture of MTZ-S sub-populations, sterile velvet blots were applied to the surface of a CBA plate of well-separated mixed culture colonies and replicate plates of this were prepared on CBA and MTZ CBA. Following incubation (microaerobically at 36°C for 48 h) and comparison of plates, MTZ-S colonies that grew on the CBA but not on MTZ CBA were identified and subcultured on CBA for storage. Purity of MTZ-S populations was confirmed by demonstration of an inability to grow on MTZ CBA. Stock cultures of all sub-populations were prepared as described in section 2.1.2.

## 2.9 Extraction of DNA from bacterial culture

DNA was prepared by a described method (Wilson 1987) that uses cetyltrimethyl-ammonium-bromide (CTAB) and organic solvents phenol and chloroform, to separate genomic DNA of lysed cells from other contaminating cellular components such as protein and polysaccharide.

Briefly, bacteria, cultured as stated in section 2.1.2, were harvested and suspended in 900 µl saline (0.85 % *w/v*). Bacterial cells were pelleted by centrifugation (13 000 *g* for 5 min), and resuspended in 500 µl TE buffer - 0.1M Tris, 0.05M EDTA, pH 8.0 [Sigma Ltd]. Cells were lysed and contaminating protein degraded by the addition of 30 µl SDS (10 % *w/v*) [BDH] and 5 µl proteinase K (25 mg/ml) [Sigma Ltd] respectively. The solution was mixed and incubated at 37 °C for 1 h until lysis was complete. DNA was retained in solution by the addition of 100 µl of NaCl (5 M) [BDH], and 80 µl of CTAB solution (10 % *w/v* CTAB in 0.7 M NaCl [Sigma Ltd]) was added to remove polysaccharide and protein contaminants. This was mixed and incubated at 65 °C for 10 min. Remaining impurities such as proteins were removed from the suspension by addition of chloroform:isoamyl alcohol (24:1 *v/v*) [Fluka Biochemica, Sigma Ltd] followed by centrifugation (13 000 *g* for 5 min). The upper aqueous layer containing DNA was transferred to a new 1.5-ml polypropylene tube [Anachem Ltd, Luton, Bedfordshire, UK], phenol:chloroform:isoamyl alcohol (25:24:1) [Fluka Biochemica, Sigma Ltd] added, mixed and the solution centrifuged (13 000 *g* for 5 min). The upper aqueous layer was transferred to a fresh tube and nucleic acid precipitated by the addition of 0.6 vol cold (- 20 °C) isopropanol [AnalR, BDH] and repeated tube-inversion. DNA was pelleted by brief centrifugation (10 000 *g* for 1 min) and washed in 1 ml of 70 % (*v/v*) ethanol [Molecular Grade, BDH] that was subsequently discarded. The DNA pellet was dried in a DNA 110 SpeedVac® vacuum drier [Thermosavant, Holbrook, New York, USA] and resuspended by incubation at 4 °C overnight in 100 µl sterile distilled water [Promega UK Ltd, Southampton, UK]. DNA was stored long-term at -20 °C.

## 2.10 Quantification of extracted DNA

DNA extracted from culture was quantified by spectrophotometry. DNA absorbs light at a peak wavelength of 260 nm, where an optical density (OD) of one is equivalent to 50 µg/ml of double stranded DNA. Contaminating protein and other molecules such as RNA, and polysaccharide frequently remain in DNA extracts and have peak absorbances at 280 nm and 230 nm, respectively. By recording absorbances at all three wavelengths, the purity of a DNA preparation can be calculated by the OD ratio at 260 nm against 230 nm and 280 nm and the DNA concentration calculated on the basis of $OD_{260}$ measurements (Sambrook *et al.* 1989).

The spectrophotometer [Eppendorf BioPhotometer, Eppendorf, Cambridge, UK] was set to zero by absorbance measurement of sterile distilled water in a sterile disposable UVette® [Eppendorf]. Extracted DNA was diluted 1/100 in sterile distilled water [Promega UK Ltd] in a UVette® and the optical density recorded at 230 nm, 260 nm and 280 nm, using the stored double stranded DNA program. DNA preparations were considered sufficiently pure if the $OD_{260/280}$ ratio was >1.8 and the $OD_{260/230}$ ratio was ≥2.0.

## 2.11 DNA Extraction from human biopsies

Three methods for DNA extraction from human biopsies were examined in this study.

### 2.11.1 Boiling method

DNA extractions were performed following a method described previously (Bickley *et al.* 1993). Biopsies were suspended in 300 µl sterile distilled water, agitated by vortexing for 1 min, then incubated at 100 °C for 10 min and centrifuged at 14 000 *g*.

### 2.11.2.1 Digestion method

Genomic DNA was extracted from human gastric, bladder and colonic biopsies using a modification of a described method (Marais *et al.* 1999). Briefly, biopsies were homogenised in Griffith's tubes [Sigma Ltd] containing 400 µl sterile saline (0.85 % *w/v*), transferred to 1.5 ml tubes [Anachem] centrifuged (10,000 *g* for 2 min) and the supernatant discarded. Pellets were resuspended in extraction buffer (20 mM TrisHCl [Sigma Ltd], pH 8.0, 0.5 % *v/v* Tween20 [BDH]) and proteinase K [Sigma Ltd] (0.5 mg/ml), vortexed (5 s) and incubated at 56 °C for 1 h, then 100 °C for 10 min [Intelligent Heating Block, Hybaid Ltd, Ashford, Middlesex, UK]. DNA extracts were stored ( –20 °C) until required.

### 2.11.2.2 Sterilisation of Griffith's tubes

Homogenisation tube components were sterilised after use by heating to 121 °C for 15 min in an autoclave [Cabburn Sterilisers, Denley, Thermo Life Sciences, Basingstoke, Hampshire, UK]. Tubes were then washed with hot water and detergent, to remove residual biopsy material, rinsed in molecular grade water and immersed in depurinating solution (1 M HCl, [BDH]) for 1 h to degrade any potentially contaminating DNA. Following three washes in molecular grade water, tubes were air dried, wrapped in aluminium foil and sterilised by incubation at 160 °C for 2 h in a hot air oven [Sanyo Gallenkamp, Loughborough, Leicestershire, UK].

### 2.11.3 Spin column method

It is well recognised that various blood components are potent inhibitors of *Taq* polymerase (Wilson 1997). As bronchial biopsies are highly vascular, DNA was extracted from these according to the manufacturer's protocol [QIAGEN Ltd,

Crawley, West Sussex, UK] using the commercial QIAmp® DNA Mini Kit, designed specifically for extraction of tissue and blood products.

### 2.12 DNA extraction from stool specimens

Faecal specimens contain many substances that inhibit *Taq* polymerase and thus the PCR reaction (Wilson 1997). The chosen method of DNA extraction should aim to remove as much of these as is possible while still retaining high yields of template. The following methods were tested to establish the optimal method for extraction of *H. pylori* DNA.

#### 2.12.1 Guanidium isothiocyanate/silica (or diatoms)

The chaotropic agent guanidium isothiocyanate (GuSCN) not only lyses cells but also inactivates nucleases - thus its use in DNA and RNA extraction is widespread (Pitcher *et al.* 1989). DNA binds silica or diatomaceous particles (diatoms) in the presence of high GuSCN concentrations and this approach was first described for DNA extraction from clinical samples by Boom *et al* in 1990. The two variations of the original method described, using either silica or diatoms, were evaluated during this study – as was a further modification where the GuSCN buffer L6 contained milk to block binding of inhibitory substances to the silica or diatoms (Boom *et al.* 1999).

DNA was extracted from faeces using a modification of the method described (Boom *et al.* 1990). Briefly, 100-200 µl sample was added to 900 µl L6 buffer – 10 M GuSCN, 0.1 M Tris HCl, pH 6.4, 35 mM EDTA, 2 % (*w/v*) Triton X-100 [Severn Biotech Ltd, Kidderminster, Worcestershire, UK] – or modified L6 buffer containing skimmed milk powder (50 mg/ml) [Marvel] (Boom *et al.* 1999) and incubated at room temperature for 5 min. Insoluble faecal particles were sedimented by brief centrifugation (12000 *g* for 15 s), the supernatant transferred to a fresh 1.5 ml tube

[Anachem Ltd] and nucleic acids removed from solution by the addition of 100 µl coarse silica [Severn Biotech Ltd] or diatoms [Celite, Sigma Ltd UK] followed by vortexing and agitation at room temperature for 10 min. The silica (or diatoms) was pelleted by centrifugation (12 000 *g* for 15 s) and washed twice with 1 ml L2 buffer – 10 M GuSCN 0.1 M Tris-HCl, pH 6.4 - [Severn Biotech Ltd], washed twice with 1 ml cold ethanol (80 % *v/v*) [Molecular Grade, BDH] then once with 1 ml cold acetone [AnalR BDH]. The pellet was dried (55 °C for 15 min), and nucleic acids dissociated from the silica/diatoms by incubation in 150 µl sterile distilled water [Promega UK Ltd] (55 °C for 5 min). The extracted DNA was transferred, following brief centrifugation, to a 0.5-ml tube [Anachem Ltd] and stored at –20 °C until required.

### 2.12.2 Spin Columns

Spiked stools were extracted by the commercial QIAamp® DNA Stool Mini Kit [QIAGEN Ltd], following the manufacturer's protocol. The principle of this kit is similar to that of the method described in section 2.12.1, based on binding of DNA to silica in the presence of chaotropic salt. However the DNA is bound to a silica gel membrane in a mini-column format that facilitates the multiple rapid washing steps required before final DNA elution. Pre-treatment of specimens with the InhibitEX kit component allows adsorption of substances that may inhibit PCR or degrade DNA.

### 2.12.3 Automated DNA extraction (MagNA Pure)

The MagNA Pure system [Roche Diagnostics Ltd, Lewes, East Sussex, UK] is an automated means of DNA extraction that is based on similar principles to the Boom method, although guanidine hydrochloride is used instead of GuSCN. Bacterial cells are lysed and DNA stabilised by chaotropic salts and detergent, but an additional proteinase K step is included to degrade protein contaminants. Nucleic acids are then

bound to the silica surface of magnetic glass particles and these are subsequently retained during a series washing steps by the application of a magnet to the reaction cartridge (Figure 2.1). Nucleic acid is subsequently eluted at an elevated temperature in elution buffer [Roche Diagnostics Ltd] and transferred to a cooling block. All processes are performed robotically, as illustrated in Figure 2.1.

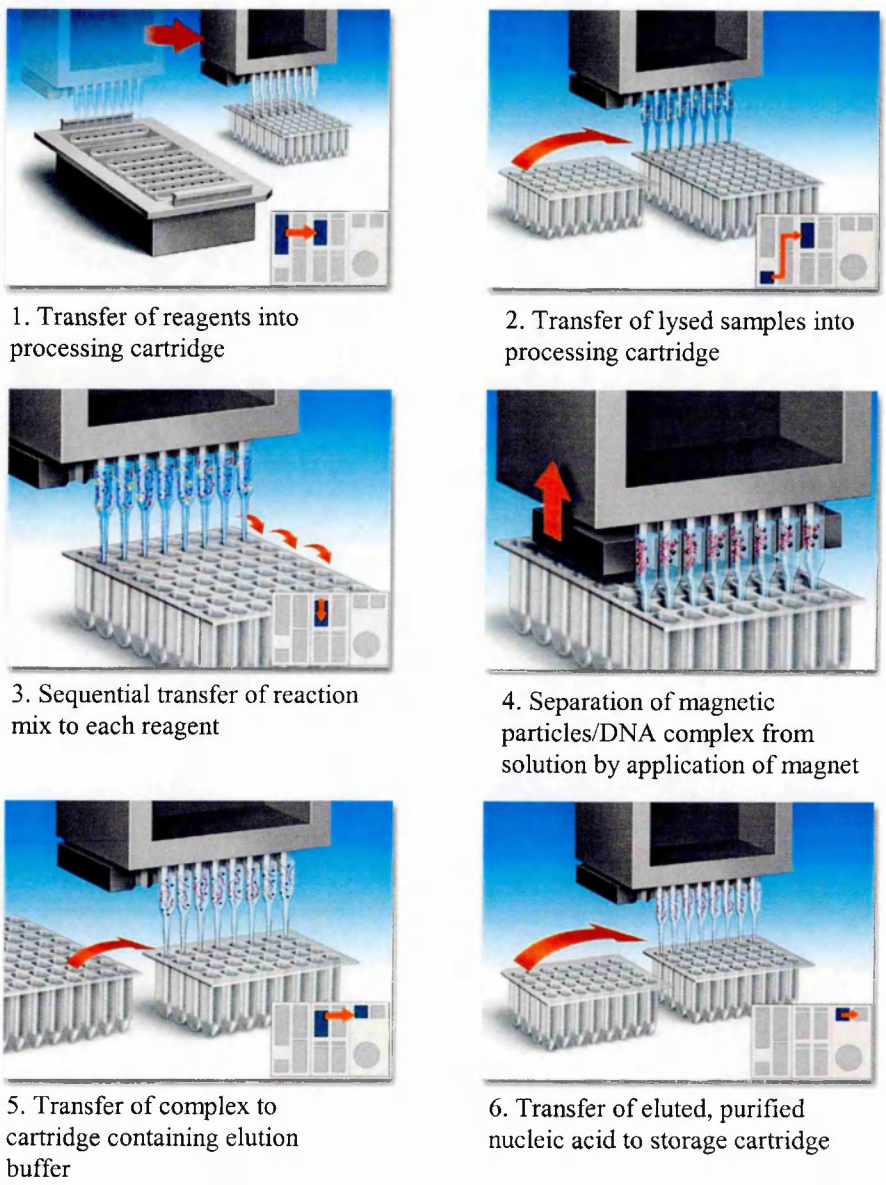



**Figure 2.1 Illustration of the stages of nucleic acid extraction on the MagNA Pure System** [Roche] (adapted from www.roche-applied-science.com)

Two kits based on this principle, the MagNA Pure LC Total Nucleic Acid Isolation Kit and the MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) [Roche Diagnostics Ltd], were evaluated for DNA extraction from stool samples. However, for the latter kit, specifically for bacterial DNA extraction, an additional pre-lysis step that included proteinase K digestion and heating of specimens (65 °C and 95 °C for 10 min each) was required prior to loading samples onto the MagNA Pure instrument [Roche Diagnostics Ltd] for automated extraction. Aliquots (200 µl) of artificially seeded stool suspensions (10 % *w/v*) prepared in BB or in BB-milk [Marvel] solution (330 mg/ml) were extracted according to the manufacturer's protocol for each kit.

## 2.13 Removal of PCR inhibitors

Faecal DNA extracts were processed by additional methods for further removal of PCR inhibitory substances.

### 2.13.1 Dilution

All faecal DNA extracts were diluted 1/10 in sterile distilled water [Promega UK Ltd] for PCR analysis. DNA extracted by the MagNA Pure LC DNA Isolation Kit III (Bacteria Fungi) [Roche Diagnostics Ltd] (section 2.12.3) were diluted 1/100 also.

### 2.13.2 Polyvinyl Pyrrolidone (PVP) treatment

PVP has been shown to remove some PCR inhibitors from faecal DNA extracts, probably by forming hydrogen bonds with phenolic compounds and other charged molecules (Lawson *et al.* 1997).

Faecal DNA extracts in this study were PVP-treated following the protocol described previously (Lawson *et al.* 1997). DNA extracts (50 - 150 µl) were mixed with 150 µl PVP/TE (10 % w/v) [Sigma Ltd] and incubated at room temperature for

10 min. 100 µl ammonium acetate (2 M) [BDH] was added, mixed by vortexing (5 s) and DNA precipitated by incubation at –20 °C in 600 µl isopropanol [AnalR BDH] for 20 min followed by centrifugation (13000 *g* for 10 min). After the supernatant was discarded, the DNA pellet was dried in a DNA 110 SpeedVac® vacuum drier [Thermosavant], reconstituted in 50 µl sterile distilled water and stored at –20 °C until further use.

## 2.14 Conventional PCR assays

PCR is a rapid, simple means of amplifying a specific gene to generate many thousands of copies. Specific oligonucleotide primers that flank the gene (or gene fragment) of interest bind to complementary sequences on the DNA template, allowing thermo stable DNA polymerase to extend the copy strand. This reaction is repeated 30 - 40 times by thermal cycling, amplifying the target gene. A detailed explanation of PCR is provided in section 1.6.

### 2.14.1 Precautions against PCR contamination

Contamination of PCR reactions with extraneous PCR product (amplicon) or DNA can generate falsely positive results. All PCR reactions carried out in this study were subject to strict precautions to minimise this. The workflow was designed so that reactions were performed in a direction of low- to high-level risk of amplicon contamination, using designated pipettes and plastic consumables at each stage – sterile RNase and DNase free filtered tips were used throughout [RAININ Instrument Co. Inc, Leiden, The Netherlands]. PCR master mixes were prepared in a room free from DNA and PCR amplicons. Template DNA was then added in a separate laboratory where all DNA extractions had been performed but no amplicons had been

handled. Thermal cycling and amplicon analysis was carried out in a separate designated laboratory. Template for the second round of nested PCR was added in a room separate from the area of routine amplicon analysis. In addition, all PCR experiments included a negative control tube containing sterile distilled water instead of template to ensure that amplicons were only generated from specific template DNA and not from extraneous contaminants.

### 2.14.2 Primer design

Primers were designed to target conserved regions of specific genes, identified by multiple gene alignments. Although primer sequences were limited in some cases by the availability of such conserved regions, where possible the basic rules of primer design were adhered to. Namely, primers ranged in length from 15 – 30 bp, did not contain long runs of a single base or internal complementary sequences that could lead to secondary structure. Primers were not complementary to each other, so avoiding potential for primer dimer formation, and unbalanced G/C and A/T distributions were avoided, as was an overall low G + C content. As amplification starts at the 3' end of the primer, this is the most important end for specific priming. The 3' end was designed to minimise the possibility of secondary structure, or repetitive or palindromic sequence and primers were designed so that any unavoidable mismatches with the template, due to intrinsic heterogeneity, were positioned close to the 5' end. Primer pairs were designed to have similar melting temperatures (Tm). Many different formulae are available to calculate primer Tm. In this study the formula Tm = 4 °C(G + C) + 2 °C(A + T), that is simple and accurate for Tm calculation of primers in the range 18 – 24 bp was applied (Suggs *et al.* 1981).

### 2.14.3 Conventional block PCR reactions

PCR master mixes were prepared in a final reaction volume of 50 µl as described in Table 2.1, unless stated otherwise. Volumes per reaction were multiplied by the number of samples tested plus two extra reactions for an appropriate positive control (specific DNA) and for a negative control (distilled water). The manufacturers of individual PCR reagents stated in this section were used throughout this study. All PCR reactions were carried out in sterile RNase and DNase-free thin-walled 0.5-ml Thermo tubes [ABgene, Epsom, Surrey].

**Table 2.1 Reaction conditions for conventional block PCR assays**

| Reagent [Manufacturer] | Concentration | Volume (µl) | Final Concentration |
|---|---|---|---|
| Sterile distilled water | | 31.3 | |
| PCR Buffer* [Invitrogen] | 10 X | 5.0 | 1 X |
| dNTPs [Invitrogen] | 2 mM each | 5.0 | 200 µM each |
| $MgCl_2$ [Invitrogen] | 50 mM | 1.5 | 1.5 mM |
| Primer [MWG†] | 20 µM each | 1.0 | 0.4 µM each |
| *Taq* polymerase [Invitrogen] | 5 U/µl | 0.2 | 1 U |
| Template DNA | 20 ng/µl | 5 | 100 ng‡ |

*1 X PCR buffer is 20 mM TrisHCl, pH 8.4, 50 mM KCl, 0.2 % v/v glycerol.
†[MWG Biotech UK Ltd, Milton Keynes, UK].
‡5 µl DNA extract from clinical samples added (specific DNA concentration not known).

Unless otherwise stated, all reaction mixes were held for 5 min at a denaturation temperature of 95 °C and target genes amplified by 35 cycles of denaturation at 95 °C for 30 s, annealing for 30 - 60 s at an annealing temperature (Ta) based on the melting

temperature (Tm) of the specific primer, and elongation at 72 °C for 45 - 60 s. Strand extensions were completed by a final 5-min incubation at 72 °C. Thermal cycling for detection assays (section 2.16) were carried out in a PCR Sprint thermal cycler [Hybaid Ltd]. With the exception of the *vacA* genotyping multiplex assay (section 2.18.1.2) that was performed in a DNA Engine [MJ Research, Genetic Research Instrumentation Ltd, Braintree, Essex], all other assays described in sections 2.15 - 2.18 were performed in both models of thermal cycler.

### 2.14.4 PCR modifications for analysis of highly inhibitory specimens

Binding of inhibitory substances to *Taq* DNA polymerase can be blocked by the inclusion of Bovine Serum Albumen (BSA) in the PCR reaction mix. In cases where specimens are particularly inhibitory, *Taq* polymerase can be replaced with *Tth* DNA polymerase that is less sensitive to such effects. Combination of these approaches has been shown to reduce the effects of PCR inhibitors in a range of specimens including stools (Abu al-Soud and Radstrom 1998). PCR reactions were prepared as described in Table 2.2 but were otherwise processed as stated in section 2.14.3. These modified conditions were used for analysis of DNA extracted from stools by the MagNA Pure LC DNA kit III (Bacteria) [Roche Diagnostics Ltd] (section 2.12.3) that had been further processed as described in section 2.13.

**Table 2.2 Reaction conditions of PCR using *Tth* polymerase**

| Reagent [Manufacturer] | Concentration | Volume (µl) | Final Concentration |
|---|---|---|---|
| Sterile distilled water | | 29.6 | |
| Chelate PCR Buffer* [Promega Ltd] | 10 X | 5.0 | 1 X |
| dNTPs [Invitrogen] | 2 mM each | 5.0 | 200 µM each |
| $MgCl_2$ [Promega Ltd] | 50 mM | 2.5 | 2.5 mM |
| Primer [MWG] | 20 µM each | 1.25 | 0.5 µM each |
| BSA, acetylated [Promega Ltd] | | 0.2 | 0.4 % |
| *Tth* polymerase [Promega Ltd] | 5 U/µl | 0.2 | 1 U |
| Template DNA (faecal extract) | | 5 | |

*1 X chelate buffer is – 10mM Tris-HCl, pH 8.3, 100 mM KCl, 0.75 mM EGTA, 0.05 % (*v/v*) Tween 20 and 5 % (*v/v*) glycerol

### 2.14.5 Applications of conventional block PCR

In the course of this study, PCR was applied in the following four main areas:

i) Evaluation of DNA extracts from clinical samples for residual substances inhibitory to the PCR reaction.

ii) Detection of species of *Helicobacter* from a range of clinical samples.

iii) Investigation of *H. pylori* antibiotic resistance genes.

iv) Investigation of virulence potential of *H. pylori* strains.

Details of the assays applied will be provided in subsequent sections 2.15 – 2.18.

## 2.15 Internal control PCR

PCR can be inhibited by a wide range of substances found in clinical specimens, such as haem, urea or bile acids and salts (Wilson 1997). Failure of an extraction method to remove such material from a DNA preparation could potentially lead to the

generation of false-negative results. Internal control PCR assesses extracted samples for presence of PCR inhibitors. Primers were selected to amplify an endogenous target that was always present in the specimen, regardless of patient disease status. Specific amplicon should always be generated, and failure to do so indicates presence of residual PCR inhibitors in the specimen extract.

### 2.15.1 Human mitochondrial PCR

All tissue specimens of human origin will contain the human mitochondrial cytochrome oxidase subunit 3 gene. DNA extracts from human gastric, bladder, colonic and bronchial biopsies were initially tested by a PCR assay using the primer pair H6A and H6B (Table 2.3) that amplifies this target (Cadieux *et al.* 1993), as an internal control to ensure that extraction methods applied were sufficient to remove PCR-inhibitory substances.

### 2.15.2 Universal prokaryote-specific PCR

DNA extracts from stool specimens contain large amounts of nucleic acid from a wide range of bacterial species, as well as numerous PCR inhibitors. The primer pair Epsilon (ε) and 1510 (Table 2.3) target a region of the 16S rRNA gene that is universally conserved in bacterial species (Gibson and Owen 1998). All stool DNA extracts were analysed by this internal control assay, to compare efficacy of different extraction methods and post-extraction modifications described in sections 2.12 and 2.13, respectively, for removal of PCR inhibitors.

**Table 2.3 Details of primers, annealing temperatures (Ta) and amplicon sizes of internal control PCR assays for analysis of human biopsy and stool specimen DNA extracts.**

| Target gene | Primer name | Sequence (5′ → 3′) | Ta (°C) | Amplicon size (bp) |
|---|---|---|---|---|
| Human tissue-specific | | | | |
| Cytochrome oxidase | H6A<br>H6B | ATG ACC CAC CAA TCA CAT GCC TAT CA<br>ACT AGT TAA TTG GAA GTT AAC GGT ACT A | 55 | 823 |
| Universal prokaryote-specific | | | | |
| 16S rRNA | ε<br>1510 | AAG AGT TTG ATC ATG GCT CAG<br>GGT TAC CTT GTT ACG ACT T | 50 | 1462 |

## 2.16 PCR detection assays

Thirteen PCR detection assays amplifying five different target genes were applied in the course of this study. These were either uniplex, single-round format (n = 9), nested format (n = 3) or multiplex single-round format (n = 1). The principles of these different formats have been described earlier (section 1.6). Assays were selected, or new tests designed, according to the required application, while different target genes were selected after consideration of the relative merits of each.

### 2.16.1 16S rRNA genes

The rationale of 16S rRNA as a potential target for genus and species-specific PCR assays has been discussed in section 1.7.1. A number of published and novel 16S rRNA specific PCR assays were applied in the course of this study as follows:

#### 2.16.1.1 *Helicobacter* genus-specific assays.

Prokaryote-specific primers C70/B37, previously applied in the characterisation of *H. bilis* (Fox *et al.* 1995), and published *Helicobacter*-specific primer pairs HGF/HGR

(Bohr *et al.* 1998) and H297F/1026R (Logan *et al.* 2000) (Table 2.4) were applied to detect helicobacters in bladder, colonic and bronchial biopsies. The latter PCR assay, described by Logan *et al* (2000), was also applied to stool DNA extracts in both a single-round format and as the first round of a nested PCR assay (section 2.16.1.3).

**2.16.1.2 *H. pylori*-specific PCR assay (single round format)**

The published primers Hp1 and Hp2 (Ho *et al.* 1991) (Table 2.4) were initially applied to gastric biopsies and the performance compared with other *H. pylori* specific assays. This assay was subsequently applied to bladder, colonic and bronchial biopsies and to stool samples.

**2.16.1.3 *H. pylori*-specific PCR assay (nested format)**

Stool DNA extracts were examined for *H. pylori* by combining two assays targeting 16S rDNA in a nested format. In the first PCR round, the Logan assay described in section 2.16.1.1 (primer pair H277F/H1026R, Table 2.4) was applied to amplify *Helicobacter*-specific DNA. The resultant amplicon was the template for the second PCR round using the *H. pylori*-specific Ho assay (primer pair Hp1/Hp2, Table 2.4) described in section 2.16.1.2. To prevent carry-over of first-round primers, reaction conditions of the second PCR round were modified so that less template (0.5 µl) was added to reaction mix (49.5 µl). The volume of water added to each reaction was adjusted accordingly so that reagent concentrations remained constant.

**2.16.1.4 HHLO-specific PCR (HHLO-16)**

Primers (HeilF and HeilR, Table 2.4) for a novel PCR assay that could detect all HHLOs (defined in section 1.13.2) were designed following *in silico* alignment in GeneBase version 1.0 [Applied Maths, Kortjivik, Belgium] of 51 16S rRNA gene sequences from 19 species of *Helicobacter* held in public databases [Entrez

nucleotide, includes GenBank, RefSeq and PDB, http://www.ncbi.nlm.nih.gov] (Appendix B.2). This assay was applied to human and feline gastric biopsies, and the performance was compared with an existing published assay targeting the urease B (*ureB*) gene (section 2.16.2.2).

**Table 2.4 Details of primers, annealing temperatures (Ta) and product sizes of PCR detection assays amplifying 16S rRNA genes.**

| Assay specificity (*) | Primer name | Sequence (5′ → 3′) | Ta (°C) | Amplicon size (bp) |
|---|---|---|---|---|
| Prokaryote | C70<br>B37 | AGA GTT TGA TYM TGG C<br>TAC GGY TAC CTT GTT ACG A | 50 | 1505 |
| *Helicobacter* | HGF<br>HGR | CGC GTG GAG GAT GAA GG<br>CGT GCA GCA CCT GTT TTC | 58 | 639 |
| *Helicobacter* (n1) | H297F<br>H1026R | GGC TAT GAC GGG TAT CCG GC<br>GCC GTG CAG CAC CTG TTT TC | 58 | 749 |
| *H. pylori* (n2) | Hp1<br>Hp2 | CTG GAG AGA CTA AGC CCT CC<br>ATT ACT GAC GCT GAT TGT GC | 60 | 109 |
| HHLO† | HeilF<br>HeilR | AAG TCG AAC GAT GAA GCC TA<br>ATT TGG TAT TAA TCA CCA TTT C | 53 | 112 |

* n1 and n2 denotes primers also used in first and second round of nested PCR, respectively

†Novel primers designed for this study

### 2.16.2 Urease genes

As discussed in section 1.7.2, urease genes have frequently been targets for specific PCR assays in *Helicobacter*.

#### 2.16.2.1 *H. pylori*-specific assay (*glmM*)

As described in section 1.7.2.2, the PCR assay of Brisou *et al* (1990) was originally thought to target a urease gene. For this reason, this assay has been described in this

section, although *ureC* has since been renamed *glmM* (De Reuse *et al.* 1997). This assay was applied, using primer pair ureCF/ureCR (Table 2.5), to detect *H. pylori* in the initial phases of PCR-based analysis of gastric biopsies, and the performance compared with other *H. pylori* specific assays targeting 16S rRNA genes (section 2.16.1.2) and *vacA* (section 2.16.3.1).

#### 2.16.2.2 '*H. heilmannii*'-specific assay

A published assay using a primer pair (ureBHeilF/ureBHeilR, Table 2.5) that amplifies an *'H. heilmannii'* specific fragment of the *ureB* gene was applied following the protocol described (Neiger *et al.* 1998). PCR conditions were similar to those described in section 2.14, except that 2.5 U of *Taq* polymerase was included in each reaction and thermal cycling conditions comprised one cycle of denaturation, annealing and extension at 94 °C for 3 min, 57 °C for 2 min and 72 °C for 3 min, respectively followed by 31 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min and a single final 5-min extension at 72 °C. Performance of this assay was compared with that of the novel HHLO-16 assay (section 2.16.1.4) by application to human and feline gastric biopsies.

#### 2.16.2.3 *H. felis*-specific assay

The published *H. felis*-specific PCR assay, where primer pair ureBFelF/ureBFelR (Table 2.5) amplified regions of the *ureB* gene was performed following the stated protocol as described in section 2.16.2.2 (Neiger *et al.* 1998). This assay also was applied to human and feline gastric biopsies to evaluate the specificity of the novel HHLO-16 assay described in section 2.16.1.4.

**Table 2.5 Details of primers, annealing temperatures (Ta) and amplicon sizes of PCR detection assays amplifying urease genes**

| Target Gene | Primer name | Sequence (5′ → 3′) | Ta (°C) | Amplicon size (bp) |
|---|---|---|---|---|
| ***H. pylori* -specific** | | | | |
| *glmM* (="*ureC*") | ureCF<br>ureCR | AAG CTT TTA GGG GTG TTA GGG GTT T<br>AAG CTT ACT ACT TTC TAA CAC TAA CGC | 55 | 294 |
| ***'H. heilmannii'*-specific** | | | | |
| *ureB* | ureBHeilF<br>ureBHeilR | GGG CGA TAA AGT GCG CTT G<br>CTG GTC AAT GAG AGC AGG | 57 | 580 |
| ***H. felis*-specific** | | | | |
| *ureB* | ureBFelF<br>ureBFelR | ATG AAA CTA ACG CCT AAA GAA CTA G<br>GGA GAG ATA AAG TGA ATA TGC GT | 60 | 1150 |

### 2.16.3 Vacuolating cytotoxin (*vacA*) gene

As discussed in section 1.12.1, virtually all strains of *H. pylori* possess the *vacA* gene (Xiang *et al.* 1995). As *vacA* is found in *H. pylori* only, this is a potentially highly specific target for a PCR-based detection assay. Numerous partial and complete *vacA* gene sequences are available in public databases, facilitating design of primers. Alignment of *vacA* genes demonstrates that some regions such as the mid-region are highly diverse, but that other more conserved areas are potential regions for primer design.

#### 2.16.3.1 *H. pylori*-specific assay HpVac (single round format)

Primers vac3624F and vac3853R (Table 2.6) for this novel assay were designed by multiple alignment and analysis of the 30 complete sequences of *vacA* that were available in the public databases at the time [Entrez nucleotide] (Appendix B.1). All

alignments were performed in GeneBase version 1 [Applied Maths]. Gastric, bladder, colonic and bronchial biopsies as well as stool specimens were analysed by this assay.

#### 2.16.3.2 *H. pylori*-specific assay (nested format)

DNA extracted from stool samples were analysed by a nested-format PCR assay. Specific DNA fragments were initially amplified by primers vac3533F and vac4041R, also designed by multiple alignment of the 30 sequences listed in Appendix B.1, (Table 2.6) and 0.5 µl of the resultant amplicon was added to 49.5 µl reaction mix containing internal primers vac3624F and vac3853R (Table 2.6).

### 2.16.4 Multiplex *H. pylori* and HHLO-specific assay (HpHh)

Two assays targeting *vacA* and 16S rRNA genes, described in section 2.16.3 and 2.16.1.4, respectively, were combined to create a novel multiplex assay (HpHh) that could detect either *H. pylori* or HHLOs. PCR reactions were performed as described in section 2.14.3, except that 0.6 µM (each) primer HeilF and HeilR (Table 2.4) and 0.28 µM (each) primer vac3624F and vac4041R (Table 2.6) were added. The volume of sterile water added was adjusted accordingly to give a final reaction volume of 50 µl. Following denaturation at 95 °C (5 min), specific targets were amplified in the reaction mix through 35 cycles as follows: 94 °C for 30 s, 54 °C for 30 s and 72 °C for 45 s each; extension was completed at 72 °C for 5 min. This assay was applied to human and feline gastric biopsies as well as to human bladder, colon and bronchial biopsies. Amplicons were separated by gel electrophoresis as described in section 2.19, except that the agarose concentration was 2 % (*w/v*), to prevent diffusion of the small 112-bp HHLO fragment.

**Table 2.6 Details of primers, annealing temperatures (Ta) and amplicon sizes of novel *H. pylori*-specific PCR detection assays amplifying *vacA***

| Primer name | Sequence (5′ → 3′) | Ta (°C) | Amplicon size (bp) | *Assay Format |
|---|---|---|---|---|
| vac3624F<br>vac3853R | GAG CGA GCT ATG GTT ATG AC<br>ACT CCA GCA TTC ATA TAG A | 53 | 230 | Single-round & nested |
| vac3624F<br>vac4041R | GAG CGA GCT ATG GTT ATG AC<br>CAT TCC TAA ATT GGA AGC GAA | 54 | 417 | Multiplex |
| vac3533F<br>vac4041R | GGA GTG ATC AAT CAA GCT TGA<br>CAT TCC TAA ATT GGA AGC GAA | 53 | 509 | First round of nested |

*Different primer combinations used for assays described (sections 2.16.3 and 2.16.4)

### 2.16.5 Specific 26 kDa antigen (*ahpC*)

As discussed earlier (section 1.7.3), this 26 kDa protein was originally thought to be unique to *H. pylori* (O'Toole *et al.* 1991) and so was considered a suitable target for development of specific PCR assays (Hammar *et al.* 1992;Makristathis *et al.* 1998). The protein has subsequently been identified as an alkyl hydroperoxide reductase (encoded by gene *ahpC*) (Lundstrom and Bolin 2000) and has been found in other helicobacters, including *H. cinaedi*, *H. fennelliae* and *H. pullorum* (Lundstrom *et al.* 2001).

#### 2.16.5.1 *H. pylori*-specific assay (hemi-nested format)

DNA extracts from stools were analysed by a hemi-nested PCR assay described previously for this application (Makristathis *et al.* 1998). The first round of PCR, using primers SA-F (5' - TGG CGT GTC TAT TGA CAG CGA GC - 3') and SA-R (5' - CCT GCT GGG CAT ACT TCA CCA TG - 3') was carried out using a

modification of the original protocol (Hammar *et al.* 1992). Reagent concentrations differed from those outlined (Table 2.1) in that reactions contained 100 μM (each) dNTP, 0.1 μM (each) primer and 2.5 mM $MgCl_2$. Specific DNA fragments (281 bp) were amplified after denaturation (95 °C for 5 min) by 38 cycles of incubation at 68 °C (1 min) and 92 °C (30 s), followed by six cycles where an extension step (72 °C for 2 min) was inserted between the annealing and denaturation steps.

Amplicon (0.5 μl) from the first round PCR was added as template to the second round PCR that amplified specific DNA using SA-F and primer SA-R2 (5' - TGA TCA CTG CAT GTC TTA CTT TCA TGT TTT T - 3') according to the described protocol (Makristathis *et al.* 1998). Assay conditions differed from those described earlier (2.14.3) in that reactions contained 100 μM (each) dNTP, 0.1 μM (each) primer, 2.5 mM $MgCl_2$ and 0.5 U *Taq* DNA polymerase. Following denaturation (94 °C for 5 min), DNA fragments (209 bp) were amplified during 30 cycles of denaturation, annealing and extension at 94 °C (30 s), 68 °C (1 min) and 72 °C (45 s), respectively. Extension was completed by a final incubation at 72 °C for 5 min.

## 2.17 Conventional PCR assays for investigating *H. pylori* MTZ resistance genes

The contribution of genes *rdxA*, *frxA* and *ahpC* that encode oxygen-insensitive NADPH nitroreductase, NADPH flavin oxidoreductase and alkyl hydroperoxide reductase, respectively, to MTZ resistance was examined in a strain set comprising 13 paired isolates recovered from dyspeptic patients before and after specific eradication therapy, four isolates recovered pre-treatment from the gastric antrum of patients and

four patient isolate pairs cultured from the gastric antrum and the gastric corpus, pre-treatment. These genes were also analysed in the separated MTZ-S and MTZ-R sub-populations (section 2.8.2) observed in 11/21 patients. Thus, 50 isolates were investigated in total.

### 2.17.1 *rdxA* PCR

A 686-bp fragment of DNA containing all 630 bp of the *rdxA* gene was amplified from DNA of 50 isolates from 21 patients by the primer pair rdxF863 (modified from a primer of Jenks *et al.* (1999) and the novel primer rdxR1544 (Table 2.7). Primer rdxR1544 was designed for this study by multiple alignment of six *rdxA* gene sequences (Appendix B.4) in GeneBase version 1 [Applied Maths] held in public databases [Entrez nucleotide] at the time. All amplicons were purified by the protocol described for the Wizard® PCR Preps kit [Promega UK Ltd] in section 2.22.1. *rdxA* PCR products were either quantified and sequenced in-house (n= 23) following the method described in section 2.22 or were sequenced commercially by companies MWG Biotech Ltd [Germany] (n=11) and Cytomyx [Cambridge, UK] (n=16).

### 2.17.2 *frxA* PCR

All 651 bp of *frxA* was sequenced by PCR amplification of two overlapping fragments of the gene. Different combinations of the primers pairs EFR-1/BFR-3 and EFR-2/BFR-4 (Kwon *et al.* 2001), frxA-F/frxA-R (Jeong *et al.* 2001) and novel primers frxA2-F/frxA2-R (Table 2.7) were necessary to amplify PCR products in 48 isolates. The latter primer pair was designed later in the study, by multiple alignment of 16 *frxA* genes that had been sequenced earlier. The two fragments of *frxA* were amplified by primers EFR-1/BFR-3 and EFR-2/BFR-4 in 30 isolates, by primers frxA-F/BFR-3

and EFR-2/BFR-4 in 13 isolates, by primers EFR-1/BFR-3 and frxA2-f/frxA2-R in three isolates and by primers EFR-1/BFR-3 and EFR-2/frxA-R in two isolates. The same Ta was used during thermal cycling for all primer combinations (Table 2.7). All PCR products were purified in MultiScreen®-PCR plates [Millipore Ltd, Watford, UK], quantified and sequenced following the protocols described in section 2.22.

### 2.17.3 *ahpC* PCR

A 542-bp fragment of the 591-bp *ahpC* gene was amplified in all 50 strains from 21 patients by the primer pair H26F/H26R (Table 2.7) described previously (Lundstrom *et al.* 2001). All products were purified in MultiScreen®-PCR plates [Millipore Ltd], and sequenced as described in section 2.22.

**Table 2.7 Details of primers, annealing temperatures (Ta) and amplicon sizes of PCR assays used to investigate the contribution of *rdxA*, *frxA* and *ahpC* to MTZ resistance in *H. pylori***

| Target gene | Primer name | Sequence (5′ → 3′) | Ta (°C) | Amplicon size (bp) |
|---|---|---|---|---|
| *rdxA** | rdxF863<br>rdxR1544 | TTA GGG ATT TTA TTG TAT GCT A<br>TCA CAA CCA AGT AAT TGC ATC AA | 53 | 686 |
| *frxA* | EFR-1<br>BFR-3 | TCT CAA GCG GAA AAA TCC GG<br>AAT TTT TGA TGA TTT GAG CG | 48 | 445 |
| *frxA* | EFR-2<br>BFR-4 | ATT ATG ACA CTA ATT CTA GG<br>CTA ACG CCA AGC TTT TTA TG | 48 | 388 |
| *frxA* | FrxA-F<br>FrxA-R | GGA TAT GGC AGC CGT TTA TCA TT<br>GAA TAG GCA TCA TTT AAG AGA TTA | 48 | 780 |
| *frxA** | FrxA2-F<br>FrxA2-R | AGG TTC GCT CAA ATC ATC A<br>TTC AAT CAC TTC ATA AAT AAC | 48 | 315 |
| *ahpC* | Hp26F<br>Hp26R | TTA GTT ACA AAA CTT GCC CC<br>GCT TTC ATC CCT TTA TCG CC | 53 | 542 |

*Novel PCR primers designed for this study

## 2.18 Conventional PCR assays for investigation of *H. pylori* virulence potential

Two loci, *vacA* and *cagA*, that may be associated with *H. pylori* virulence were examined in this study.

### 2.18.1 Vacuolating cytotoxin (*vacA*)

As discussed in section 1.12.1, *vacA* has a mosaic structure comprising two families of allelic variants at the signal sequence region (s1, s2) and at the mid-region (m1, m2) (Atherton *et al.* 1995). Some specific combinations of these families (*vacA* genotype) may enhance virulence potential of *H. pylori* (Atherton *et al.* 1995).

#### 2.18.1.1 *vacA* genotype determination (uniplex format)

Signal and mid-regions of *vacA* were amplified from culture or gastric biopsy extracts in separate uniplex assays by primer pairs VAI-F/VAI-R and VAG-F/VAG-R (Table 2.8), respectively that had been described previously (Atherton *et al.* 1999).

#### 2.18.1.2 *vacA* genotype determination (multiplex format)

The primers used in separate assays (section 2.18.1.1) were combined in a novel multiplex format and conditions optimised to allow co-amplification of both regions of *vacA*. PCR reagent concentrations were as described (section 2.14.3), except that $MgCl_2$ concentration was raised to 2.0 mM, and concentrations of primer pairs VA1-F/VA1-R and VAG-F/VAG-R were 0.3 μM (each) and 0.48 μM (each), respectively. PCR products were separated by gel electrophoresis as described in section 2.19, except that the agarose concentration was 2 % (*w/v*), to improve separation of the different amplicon sizes.

### 2.18.2 Cytotoxin associated gene (*cagA*)

Approximately 70 % of *H. pylori* strains possess the *cagA* gene (Owen *et al.* 2001). This study investigated the potential involvement of this gene in bacterial virulence.

### 2.18.2.1 Amplification of *cagA* from gastric biopsies

*H. pylori*-positive gastric biopsies were screened for the *cagA* gene by a PCR assay described previously using primer pair D008/R008 (Table 2.8) (Covacci and Rappuoli 1996). Following a 5-min incubation at 94 °C fragments of *cagA* were amplified by 38 cycles of denaturation, annealing and extension at 94 °C (1 min), 60 °C (1 min) and 72 °C (1 min), respectively. A final cycle of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 5 min completed the reaction (Covacci and Rappuoli 1996).

### 2.18.2.2 Amplification of *cagA* tyrosine phosphorylation motif (TPM) regions

Three regions of the *cagA* gene, proposed to contain the putative TPMs A, B and C (Odenbreit *et al.* 2001) discussed in section 1.12.2, were amplified by novel primer pairs MotAF/TPMAR, MotBF/MotBR and MotCF/MotCR, respectively (Table 2.8). Twenty-six TPM A, two TPM B and nine TPM C amplicons were purified, quantified and sequenced following the protocol described in section 2.22. TPM B fragments from four other strains that had given equivocal results by the assay described in section 2.20.7 were sequenced commercially [MWG Biotech Ltd].

**Table 2.8 Details of primers, annealing temperatures (Ta) and amplicon sizes of PCR assays used to investigate *vacA* and *cagA* as markers of *H. pylori* virulence potential**

| Target gene | Primer name | Sequence (5′ → 3′) | Ta (°C) | Amplicon size (bp) |
|---|---|---|---|---|
| *vacA* | VAGF<br>VAGR | CAA TCT GTC CAA TCA AGC GAG<br>GCG TCA AAA TAA TTC CAA GG | 53 | 570/645 |
| *vacA* | VA1F<br>VA1R | ATG GAA ATA CAA CAA ACA CAC<br>CTG CTT GAA TGC GCC AAA C | 53 | 259/286 |
| *cagA* | D008<br>R008 | ATA ATG CTA AAT TAG ACA ACT TGA GCG A<br>TTA GAA TAA TCA ACA AAC ATC ACG CCA T | 60 | 298 |
| *cagA** | CagMotAF<br>TPMAR | GAT AGG GAT AAC AGG CAA G<br>CCT GCA AAA GAT TGT TTG GC | 53 | 356 |
| *cagA** | CagMotBF<br>CagMotBR | AAC CCT AGT CGC TAA TGG<br>GCA ACT TGA GCG TAA ATG G | 51 | 216† |
| *cagA** | CagMotCF<br>CagMotCR | CAA GCG GTA TCA GAA GCT A<br>TTA ATG CGT GTG TGG CTG TT | 53 | 179 |

*Novel PCR primers designed in this study

†Product size varied as some strains have insertions in this region of the *cagA* gene

## 2.19 DNA electrophoresis

DNA fragments and amplicons were separated by agarose gel electrophoresis and visualised by ethidium bromide staining. The DNA molecule possesses a net negative charge and will therefore migrate towards the positively charged anode in an electric field. Rate of migration of a molecule is inversely proportional to its mass, so smaller DNA fragments of faster mobility can be separated from larger molecules. Mobility of DNA during electrophoresis is also affected by temperature, ionic strength, voltage applied, and by the concentration and pore size of the separating matrix. The mobility of molecules in an individual gel can be calibrated by the inclusion of a molecular weight standard comprising DNA fragments of known size. Size of unknown DNA

can therefore be determined by comparison with standard fragments' mobility (Sambrook *et al.* 1989).

Unless stated otherwise, all DNA fragments and amplicons were electrophoresed in a 1.0 % (*w/v*) agarose gel [UltraPure, Invitrogen]

Small (8 cm x 6cm), medium (14 cm x 11 cm) and large gels (24 cm x 20cm) were prepared by the addition of 0.3 g, 0.8 g and 2.0g agarose to 30 ml, 80 ml and 200 ml TBE Buffer (0.1 M Tris, 0.09 M boric acid, 0.001 M EDTA) [Invitrogen], respectively. Agarose was melted in a microwave oven [Deltawave, Toshiba] for 2 - 3.5 min, depending on gel size, poured into the appropriate gel casting tray [Invitrogen] on a level surface and left at room temperature until set. Well combs were removed and gels transferred to an electrophoresis tank [Horizon®58 or 20.25, Invitrogen or H5, Bethesda Research Life Technologies Inc, USA] containing TBE buffer.

DNA fragments and amplicons were mixed in a 5:1 ratio with 6 X stop solution (4.0 % *w/v* sucrose [Sigma Ltd], 0.25 % *w/v* bromophenol blue [BDH], 0.05 M EDTA, [Sigma Ltd]) and carefully added to the wells of the agarose gel. With the exception of agarose gels run for quantification purposes (section 2.22.2), 123 bp molecular weight marker [Invitrogen] was included in all gels run. Unless otherwise stated, gels were run at 100 V for 30-60 min.

Gels were stained by immersion in ethidium bromide (0.5 μg/ml) [Sigma Ltd] at room temperature for 15-30 min and photographed by digital camera on a Dual Intensity Ultraviolet Transilluminator [Ultra Violet Products, Upland, USA], for printing on thermal paper [Mitsubishi Electric Europe B.V., Hatfield, Herts, UK].

## 2.20 Real-Time PCR assays

In this study, real-time PCR was performed using two different models of LightCycler, the Idaho Technology model LC32 [BioGene Ltd, Kimbolton, UK] and the Roche model [Roche Diagnostics Ltd] (Figure 2.2). The overall design of these is summarised in Figure 2.3.


**Figure 2.2 The two models of LightCycler, by Idaho Technology and Roche.**

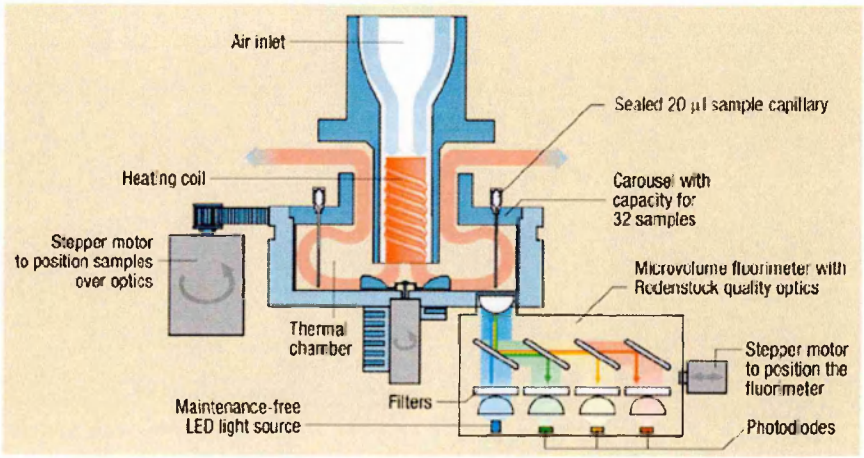



**Figure 2.3 Schematic representation of the components of the LightCycler instrument** (from the LightCycler Online Resource Site, www.roche-applied-science.com)

As real-time PCR using both models of LightCycler was applied extensively in the course of this study, a detailed explanation of the principles and applications of this technology will follow

The major difference between the Idaho model and the Roche model of LightCycler relates to the fluorimeters in each. The Idaho model measures fluorescence at two channels, channel one (F1) is optimised to detect emissions from fluorescent dyes SYBR Green 1 or fluorescein (540 nm ± 20 nm) and channel two (F2) detects emissions from Cy 5 dye (670 nm ± 20 nm). In contrast the Roche LightCycler measures fluorescence at three settings, channel one (F1) has similar settings to measure fluorescence at 530 nm ± 10 nm, but channel two (F2) and channel three (F3) are optimised to measure emissions of, respectively, LightCycler Red 640 dye at 640 nm ± 10 nm and LightCycler Red 705 dye at 710 nm ± 20 nm.

#### 2.20.1.1 The principle of rapid PCR using the LightCycler

Some real-time PCR instruments (e.g. the Taqman) use a conventional metal block for thermal cycling of reactions contained in a standard plastic microtitre plate. In contrast, LightCycler reactions are performed in glass capillaries that have a high surface area to volume ratio and instead of a metal block, thermal cycling is achieved by flushing the reaction chamber (Figure 2.3) with air of the appropriate temperature. These modifications allow rapid thermal cycling and rapid heat transfer to the sample, thereby reducing temperature transition and reaction incubation times. A major advantage of a PCR assay performed in the LightCycler is that it can be completed in as little as 30 min.

### 2.20.1.2 The principle of real-time PCR

Amplicon generation in the LightCycler can be monitored either non-specifically or specifically. SYBR Green 1 does not emit fluorescence when free in solution, but can be excited to emit a fluorescent signal at 521 nm when non-specifically bound to double stranded DNA. By monitoring the level of SYBR Green 1 fluorescence at the end of each extension stage of thermal cycling, exponential increases in levels of double stranded DNA can be identified (Figure 2.4). This approach is simple and comparatively inexpensive, but does not distinguish between specific and non-specific amplification.

Specificity can be improved by the inclusion of two fluorescently labelled hybridisation probes in the reaction mix, to monitor PCR amplification. The probes are designed to complement adjacent specific sequences within the amplicon, one probe is labelled with a 'donor' fluorophore (e.g. fluorescein) at the 3' end while the other probe is labelled with an 'acceptor' fluorophore at the 5' end (e.g. Cy5, LightCycler-Red 640). Monitoring of the PCR reaction is based on the principle that close proximity of the two dyes during hybridisation to amplicon template allows fluorescence resonance energy transfer (FRET). Emissions from the donor fluorophore, excited by the LightCycler, in turn excite the acceptor fluorophore to emit a signal, recorded by the LightCycler fluorimeter (Figure 2.5).

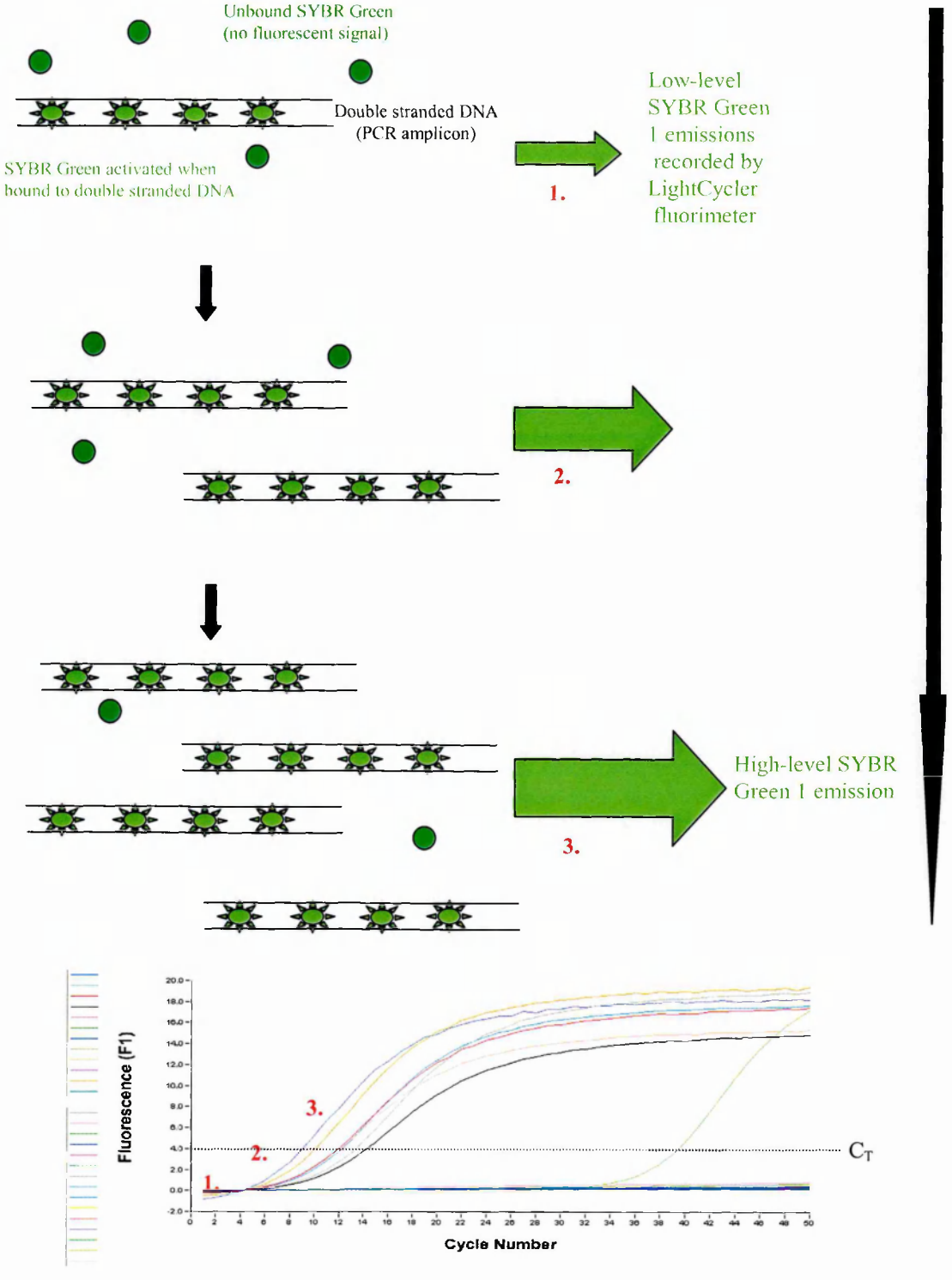



**Figure 2.4** **Schematic representation of the principle of real-time PCR in the LightCycler in relation to amplification curves generated.**

Increased amplicons levels represented graphically by increased SYBR Green 1 fluorescence. $C_T$ = Crossover Threshold.

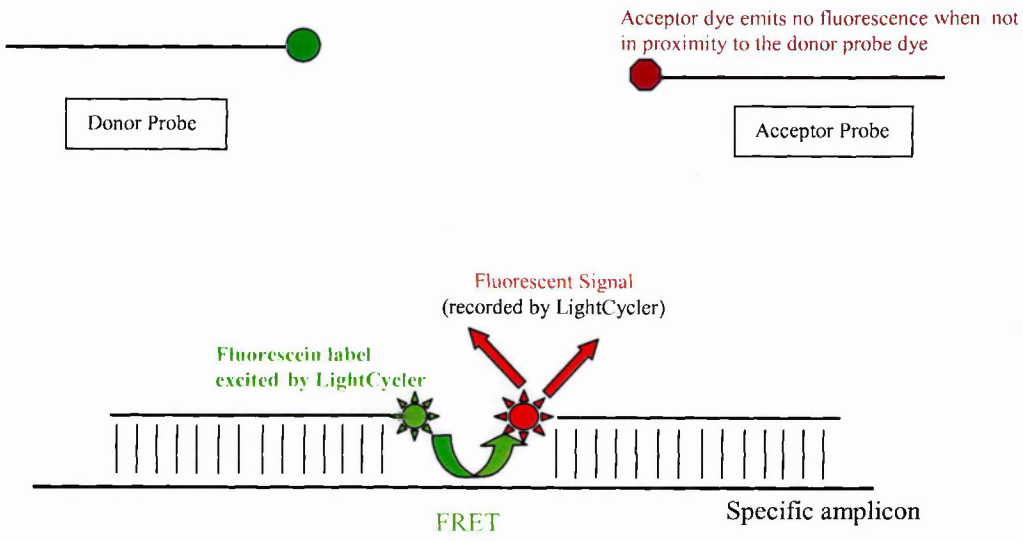



**Figure 2.5** **Schematic representation of dual probes for monitoring PCR amplification on the LightCycler.** Acceptor probe is only activated to emit fluorescence by Fluorescence Resonance Energy Transfer (FRET) when hybridised to specific template, in close proximity to donor probe.

Increased signal, recorded at each annealing stage of thermal cycling, will only occur if specific probe hybridisation to increasing amplicon levels occurs. However aside from the increased cost, one disadvantage of such an approach is that it requires prior knowledge of the amplicon internal sequence. Furthermore probes must complement areas that are highly conserved in all test subjects, which can be difficult to achieve in a highly heterogeneous species like *H. pylori*.

### 2.20.2 Further Analyses on the LightCycler

Unlike conventional PCR, most real-time PCR instruments also have the capacity for further amplicon analyses in the same reaction as the original amplification.

### 2.20.2.1 Quantification

Monitoring of amplicon generation, either specifically or non-specifically, in real time also has the advantage that it can allow quantification of the original levels of specific DNA in a sample. During the early amplification cycles, amplicon levels and fluorescence are too low to be displayed by the LightCycler software. Exponential amplification is accompanied by exponentially increasing fluorescence that is detectable by the LightCycler and is subsequently represented graphically as a plot of increased fluorescence against cycle number (Figure 2.4). The point where fluorescence first enters exponential increase, the threshold crossing point ($C_T$), (Figure 2.4) relates to the number of starting target molecules. By generating a calibration curve based on the crossing points of standards of known concentration, the level of initial target in an unknown sample can be determined. This approach has been used for quantification of both bacterial (Huang *et al.* 2001) and viral pathogens (Furuta *et al.* 1996;Stocher and Berg 2002;White *et al.* 2002). An assay for *H. pylori* quantification in gastric biopsies was described recently, based on amplification of *glmM* (He *et al.* 2002).

### 2.20.2.2 Mutation detection

The principle of mutation detection by the LightCycler is based on the altered stability of a probe-template hybridisation that results from a single base pair mismatch. By monitoring the hybridisation stability (as indicated by the dissociation temperature) of a probe spanning a sequence containing the potentially mutated bases, unknown sequences can be screened rapidly for mutations. Exact complementarity between probe and template sequence will result in a stable hybrid that has the highest dissociation temperature. Mutated sequence will lower the stability of the

hybridisation and so the probe dissociation temperature. The extent to which this temperature is lowered depends on the number, position and nature (purine or pyrimidine) of mutations in the sequence, as well as being influenced by the bases adjacent to the mutation.

Dissociation temperatures of specific probes can be determined in the LightCycler by performing a probe hybridisation melting point analysis after completion of PCR amplification. Reactions are first heated to 95 °C to denature all double stranded DNA and then cooled (40 – 50 °C) to allow specific probe hybridisation. The temperature is then slowly raised to 85 – 95 °C, while continuously monitoring fluorescence. Melting curves are plotted by calculating the first negative derivative (-dF/dT) of fluorescence versus temperature, as illustrated in Chapters 5, 7 and 10.

By labelling the probe with a fluorescent dye, changes in fluorescent emissions relating to probe dissociation can allow determination of probe melting temperature. Various different probe designs and formats are available for mutation detection, including bi-probes, dual probes, Taqman probes, molecular beacons and scorpions. However, bi-probes and dual probes are most commonly used for the LightCycler.

Bi-probes are so-named as they contain two different labels or modifications, a fluorescent dye at the 5' end (e.g. Cy 5, LightCycler Red 640, LightCycler Red 705) and a modification at the 3' end (e.g. biotin, phosphate group) to prevent spurious priming during the PCR. As illustrated in Figure 2.6, the fluorescent labels do not emit a signal when probes are free in solution, but probe template hybridisation creates double stranded DNA, allowing SYBR Green 1 excitation. The SYBR Green 1 emissions activate the probe dye by FRET, and these activated fluorescent dye emissions are recorded by the LightCycler fluorimeter. Thus probe dissociation

results in a rapid decrease in specific fluorescence (Figure 2.6). One major advantage of this format is that it requires only a single probe, reducing assay costs and complexity of optimisation. Additionally, as single probes span only a short sequence region (20 - 30 bp) it easier to design assays in poorly conserved genes. For these reasons, all the assays described and developed in this study used a bi-probe format. Bi-probe LightCycler assays have been described previously in medical bacteriology, for example to detect bacterial antibiotic resistance (Edwards *et al.* 2001;Walker *et al.* 2001) and to differentiate *Campylobacter* species (Logan *et al.* 2001).

Two (dual) probes can also be used to detect mutations. The principle of the probe dissociation monitoring is similar to that described in section 2.20.1.2, except that the acceptor probe is designed to span the mutated sequence.

In this study, real-time PCR and probe hybridisation melting point analysis on the LightCycler was applied in the detection of mutations that may confer antibiotic resistance and to investigate the potential contribution of TPMs A, B and C in *cagA* to *H. pylori* virulence. The second round of nested assays (sections 2.16.3.2 and 2.16.5.1) were adapted for the LightCycler also.

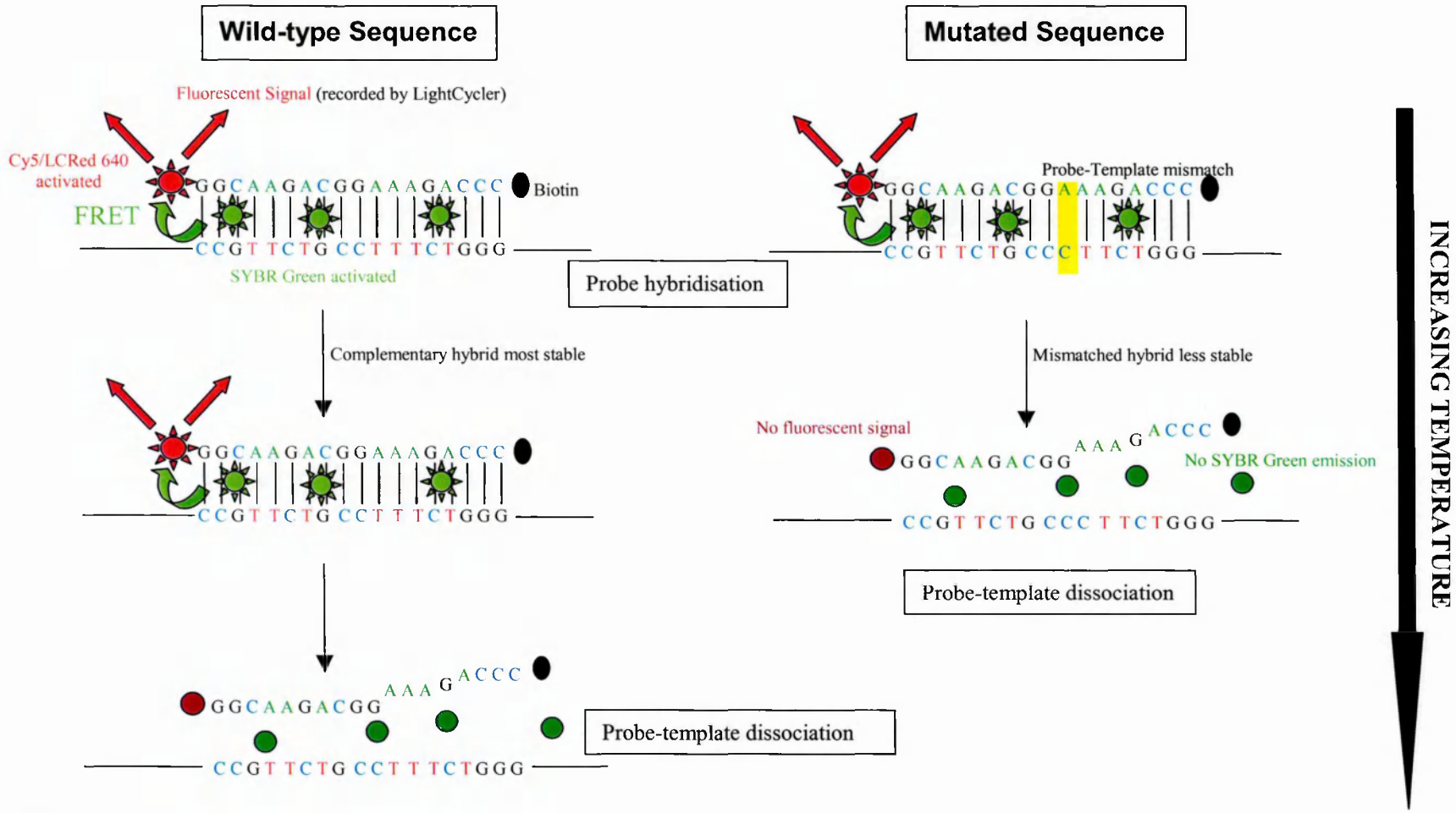



**Figure 2.6: Schematic representation of principle of mutation detection by the LightCycler (illustrated by probe 23SPr, section 2.20.1).**

The less stable mutated sequence-specific probe hybrid dissociates at a lower temperature, indicated by decreased fluorescence

### 2.20.3 Preparation of LightCycler master mix

For LightCycler assays described in sections 2.20.5 and 2.20.6, stock master mixes were prepared in advance (Table 2.9). Master mixes were aliquoted into 50 µl volumes and stored (-20 °C) until required.

**Table 2.9 Contents of LightCycler master mix**

| Reagent [manufacturer] | Concentration | Volume (µl) | Final Concentration (2x*) |
|---|---|---|---|
| Sterile distilled water | | 68 | |
| PCR Buffer† [BioGene Ltd] | 10 X | 25 | 2 X |
| dNTPs [Invitrogen] | 2 mM (each) | 25 | 400 µM |
| $MgCl_2$ [Invitrogen] | 50 mM | 5 | 6.0 mM |
| Platinum *Taq* [Invitrogen] | 5 U/µl | 2 | 0.8 U |

*Master mixes were diluted 1 in 2 for the final reaction mix described in section 2.20.4.

†Idaho technology buffer (1 X) comprises 50 mM TrisHCl, pH 8.3, 2 mM $MgCl_2$.

### 2.20.4 Preparation of LightCycler assay reactions

Reaction mixes for real-time PCR assays described in sections 2.20.5 and 2.20.6 for both models of LightCycler [BioGene Ltd and Roche Diagnostics Ltd] were prepared as described in Table 2.10, except that for the Roche model, twice the volumes described were used, giving a final reaction volume of 20 µl instead of the 10 µl used in the Idaho model.

**Table 2.10 Contents of LightCycler assay reactions**

| Reagent [manufacturer] | Concentration | Volume (µl) | Final Concentration |
|---|---|---|---|
| Master mix (Table 2.9) | 2 X | 5.0 | 1 X |
| Sterile distilled water | | 1.5 | |
| SYBR Green 1 [BioGene Ltd] | 1/1000 | 1.0 | 1/10 000 |
| Primers [MWG Biotech Ltd] | 5 µM (each) | 1.0 | 0.5 µM (each) |
| Probe [MWG Biotech Ltd] | 5 µM | 0.5 | 0.25 µM |

Subsequent processing of reactions is described in sections 2.20.5 and 2.20.6.

### 2.20.5 Determination of clarithromycin (CLA) resistance by LC-CLA assay

Point mutations in the 23S rRNA gene associated with the acquisition of CLA resistance were detected using a modification of an assay (designated LC-CLA) developed previously for the Idaho LightCycler (Gibson *et al.* 1999). Briefly, a 93-bp fragment of the 23S rRNA gene containing the two adenine residues that can mutate to confer CLA resistance was amplified and probe melting point hybridisation analysis performed. The labelled bi-probe (23SPr) sequence was complementary to the wild type CLA sensitive 23S rDNA sequence. Any resistant strains tested containing mutations A2142G, A2143G or A2142C would mismatch with this probe and lower the probe-template dissociation temperature, as described in section 2.20.2.2 and illustrated in Figure 2.6.

Reaction mixes (9 µl), prepared as stated in section 2.20.4, containing primers 23SF (5'- CAA CCA GAG ATT CAG TGA AA - 3') and 23SR (5'- GTG CTA AGT TGT AGT AAA GGT - 3') and fluorescently labelled probe 23SPr (5'- Cy5-GGC AAG ACG GAA AGA CCC-biotin - 3') were mixed in the reservoir section of a glass reaction capillary [BioGene Ltd] with 1 µl of DNA (10 ng/µl) extracted from culture

as described in section 2.9 or 1 µl of DNA extracted from gastric biopsies as described in section 2.11.2.1. Mixed reactions were then deposited in the glass capillary section of the reaction vessel by pulse centrifugation (2000 rpm) and placed in the LightCycler carousel (Figure 2.3) for amplification and probe hybridisation analysis. The thermal cycling and hybridisation conditions were as described previously (Gibson *et al.* 1999), but with the modification that amplification was increased to 75 cycles (Table 2.11). Examples of a wild-type gene, an A→G and an A→C mutation were included as controls in every run (Appendix A.2.1) to allow comparison with reactions of unknown 23S rDNA sequence.

**Table 2.11 LightCycler [BioGene] amplification and melting cycles for LC-CLA assay**

| Stage (no. of cycles) | Target temp (°C) | Incubation time (s) | Temp transition rate (°C/s) |
|---|---|---|---|
| Denaturation (1) | 94 | 20 | 20 |
| Amplification (75)* | 92 | 0 | 20 |
| | 50 | 0 | 20 |
| | 55 | 0 | 3 |
| | 72 | 5 | 20 |
| Melting (1)† | 92 | 0 | 20 |
| | 40 | 10 | 20 |
| | 90 | 0 | 0.4 |

Fluorimeter gains was set at 8 for both channel 1 and channel 2.
*Single fluorescent acquisition at the end of each extension (72 °C) stage.
†Continuous fluorescent acquisition at temperature increment (40 – 90 °C) stage.

### 2.20.6 Identification of *cagA* TPMs A, B and C.

Three novel assays were developed for the LightCycler [Roche Diagnostics Ltd] to identify strains that contained the putative *cagA* TPMs A, B or C described earlier (section 1.12.2). Multiple alignment of six complete *cagA* sequences held in public databases [Entrez nucleotide] (Appendix B.3) was carried out in GeneBase [Applied Maths]. Primer pairs were designed for each assay (Table 2.12) by selecting conserved regions of *cagA* flanking the proposed TPMs, to generate *cagA* fragments containing the relevant TPM site. The sequences of probes MotAPr, MotBPr and MotCPr (Table 2.12) were identical to the *cagA* sequences of strains that contained motifs A, B and C, respectively. Any strain that contained these motifs would have a higher melting curve peak than strains where the motif was absent. The position of primers and probes in *cagA* for each assay is illustrated in Figure 2.7.

**Table 2.12 Details of primer and labelled probes for three novel *cagA* TPM detection assays for the LightCycler [Roche Diagnostics Ltd]**

| TPM | Primer/Probe* | Sequence (5′ → 3′) |
|---|---|---|
| A | CagMotAF | GAT AGG GAT AAC AGG CAA G |
| | CagMotAR | GTG TTG ATT TTA GAC GGA TC |
| | MotAPr* | Cy5-GAA ATT TGG GGA TCA GCG TTA C-biotin |
| B | CagMotBF | AAC CCT AGT CGC TAA TGG |
| | CagMotBR | GCA ACT TGA GCG TAA ATG G |
| | CagMotBPr* | Cy5-AAT AAT GGA CTC AAA AAC AGC ACA GA-biotin |
| C | CagMotCF | CAA GCG GTA TCA GAA GCT A |
| | CagMotCR | TTA ATG CGT GTG TGG CTG TT |
| | MotCPr* | Cy5-AGC TCA AAG ATT CTA CAA AAT ACA AT-biotin |

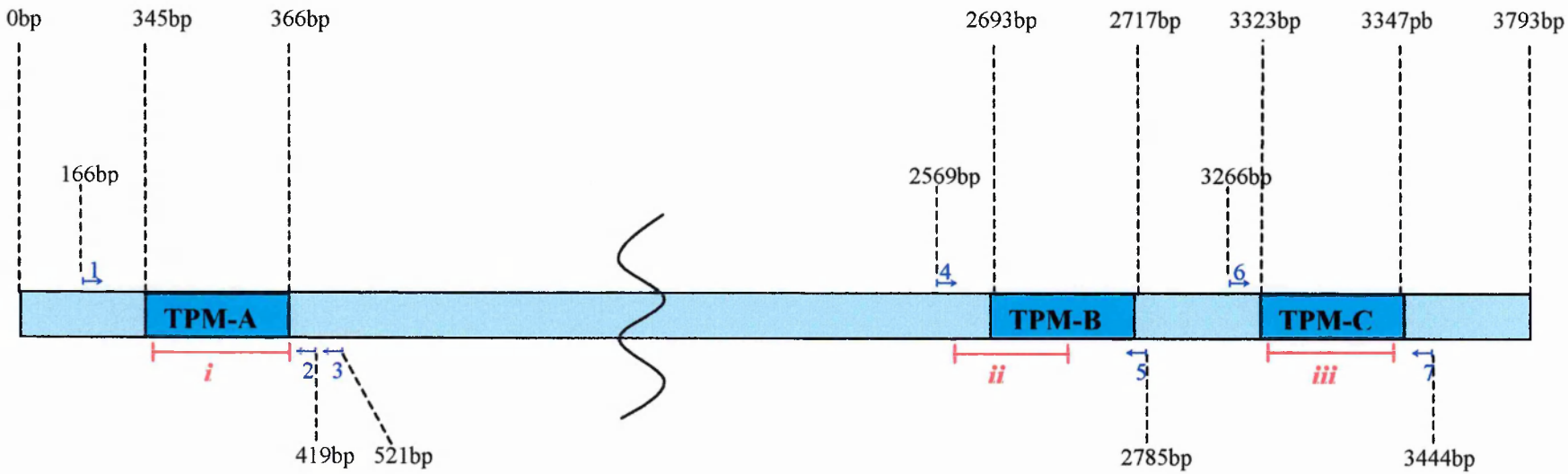



**Figure 2.7. Schematic representation of *H. pylori cagA* showing location of TPMs A, B and C; primer and probe positions for each LightCycler PCR assay and for sequencing reactions. Not to scale.**

Reaction mixes (20 µl) were prepared as stated in section 2.20.4 and contained the appropriate primers and probe for detection of either TPM A, B or C (Table 2.12). These were mixed with 2 µl template DNA (10 ng/µl) or 2 µl gastric biopsy DNA extract (prepared as described in sections 2.9 and 2.11.2.1, respectively) and then processed as described in section 2.20.5. Fragments of *cagA* were amplified by PCR and screened for specific motifs by probe hybridisation melting point analysis under the appropriate cycling programme (Table 2.13). Control strains that contained the relevant motif (Appendix A.2.3) were included in every run for comparison of melting peaks.

**Table 2.13 LightCycler [Roche Diagnostics Ltd] amplification and melting cycles for *cagA* TPM (A, B and C) detection assays**

| Stage (no. of cycles) | Target temp (°C) | | | Incubation time (s) | | | Temp (°C/s) transition rate | | |
|---|---|---|---|---|---|---|---|---|---|
| | Motif | | | Motif | | | Motif | | |
| | A | B | C | A | B | C | A | B | C |
| Denaturation (1) | | 95 | | | 10 | | | 20 | |
| Amplification (50)* | | 94 | | | 0 | | | 20 | |
| | 53 | 48 | 56 | | 0 | | | 20 | |
| | | 51 | | | 0 | | | 3 | |
| | | 72 | | | 10 | | | 20 | |
| Melting (1)† | 43 | 94 | 45 | | 0 | | 5 | 10 | 0 |
| | | 45 | | | 10 | | | 20 | |
| | | 90 | | | 0 | | 0.2 | 0.3 | 0.3 |
| Cooling | | 30 | | | 30 | | | 20 | |

Fluorimeter gains settings at each channel were F1 = 20, F2 = 15, F3 = 50.
*Single fluorescent acquisition at the end of each extension (72 °C) stage.
†Continuous fluorescent acquisition at temperature increment (45 – 90 °C) stage.

### 2.20.7 Detection of *frxA* frameshift mutation53

A novel assay (FS-53) was designed to detect a frameshift mutation in *frxA* that results from deletion of a single adenine residue at nucleotide 53, to assess the significance of this in relation to MTZ resistance. Novel primers EFR-1mod (5'- TCT CAA GCG GAA AAA TCC –3') and frxR(FS) (5'- ATC TTC TTT CAT GCG TTC A -3') targeting conserved regions of *frxA* flanking nucleotide 53 were designed following multiple alignment of the *frxA* sequences determined in the course of this study, to amplify 265-bp gene fragments. Amplicons were screened for deletion mutation 53 by melting point analysis of a labelled probe FS-53Pr (5'- LC Red 640- ATT TGC TGC AAA AAA TAC GAT C-P –3') that was designed to complement and span the mutated sequence. Amplicons containing this mutation generated a melting peak indicating a higher probe-template dissociation temperature than was observed for products lacking the deletion. This assay differed from those described in sections 2.20.5 and 2.20.6, as commercial FastStart DNA Master SYBR Green 1 master mix was used according to the manufacturer's protocol [Roche Diagnostics Ltd]. Briefly, 18-µl reactions containing, 1 X master mix [Roche Diagnostics Ltd], 6 mM $MgCl_2$, 0.5 µM each primer [MWG Biotech Ltd], 5 µM probe [TIB MOLBIOL, Berlin, Germany] were mixed with 2 µl DNA (10 ng/µl) in the cup section of the glass reaction capillaries [Roche Diagnostics Ltd], and deposited in the capillary section by pulse centrifugation (2000 rpm) [Roche Diagnostics Ltd]. Following capillary loading of the LightCycler carousel [Roche Diagnostics Ltd] PCR products were amplified and probe hybridisation melting point analysis performed following the cycling conditions described (Table 2.14).

**Table 2.14 LightCycler [Roche Diagnostics Ltd] amplification and melting cycles for FS-53 assay**

| Stage (no. of cycles) | Target temp (°C) | Incubation time (s) | Temp transition rate (°C/s) |
|---|---|---|---|
| Denaturation (1) | 95 | 600 | 20 |
| Amplification (50)* | 95 | 0 | 20 |
| | 45 | 0 | 20 |
| | 48 | 1 | 4 |
| | 72 | 5 | 20 |
| Melting (1) † | 95 | 0 | 20 |
| | 45 | 0 | 20 |
| | 85 | 0 | 0.1 |
| Cooling (1) | 30 | 30 | 20 |

Fluorimeter gains settings at each channel were F1 = 1, F2 = 12, F3 = 50.
*Single fluorescent acquisition at the end of each extension (72 °C) stage.
†Continuous fluorescent acquisition at temperature increment (45 – 85 °C) stage.

### 2.20.8 Nested PCR amplifying *H. pylori*-specific 16S rDNA

The first round of 16S rDNA amplification was conducted as described (section 2.16.1.3). Nested PCR was conducted in real-time on the LightCycler. First-round amplicon (0.5 µl) was mixed with reaction mix (19.5 µl) containing 1 X FastStart DNA Master SYBR Green 1 master mix [Roche Ltd], 3 mM $MgCl_2$ and 0.3 µM (each) primer Hp1 and Hp2 (Table 2.4). Reaction capillaries were filled by pulse-centrifugation (2000 rpm) and PCR products amplified following a 10-min incubation (95 °C), to activate hot-start *Taq* polymerase, by 50 cycles of denaturation (95 °C for 0 s), annealing (52 °C for 5 s) and extension (72 °C for 10 s). Amplicon generation was monitored by SYBR Green 1 fluorescence measurement at the end of each

extension stage. This signal was subsequently measured continuously during amplicon melting points analysis over the temperature range 45 °C to 95 °C (temperature increment 0.1 °C/s).

### 2.20.9 Nested PCR amplifying *H. pylori*-specific *ahpC*

The second round of the *ahpC* nested PCR assay was adapted to a real-time format on the LightCycler. Following first-round PCR as described in section 2.16.5.1, 0.5 µl of amplicon template was mixed with 19.5 µl reaction mix, that was identical to that described above (2.20.8) except that 0.1 µM (each) primer SA-F and SA-R2 (section 2.16.5.1) was used. Nested amplicons were generated, following a 10-min incubation at 95 °C, by 50 cycles of denaturation (94 °C for 0 s), annealing (62 °C raised to 64 °C for 3 s) and extension (72 °C for 10 s). Conditions for amplicon melting point analysis were identical to those described above (2.20.8).

## 2.21 Amplified Fragment Length Polymorphism (AFLP)

Restriction endonuclease (RE) enzymes cut DNA at specific recognition sequence sites, generating multiple fragments of varying size that can be separated by gel electrophoresis. The range and number of fragment sizes generated by this restriction fragment length polymorphism (RFLP) is sequence-dependent and can be used to distinguish organisms at the genus, species or intra-species level. RE digestion of bacterial genomic DNA can produce many fragments, leading to complex RFLP profiles that can be extremely difficult to interpret. In addition, large initial quantities of DNA are required for visualisation in a gel. AFLP is a PCR-based modification of RFLP, where, following RE digestion, specific adapter oligonucleotides are ligated to

DNA fragment ends that can then be amplified by PCR, using primers complementary to the ligated adapters. This amplification increases sensitivity of the method and thus less initial genomic material is required. By extending primer sequences one specific base downstream of the adapter sequence, amplification can be tailored so that only fragments containing the base complementary to this will be amplified. This modification reduces the number of fragments amplified and thereby simplifies the profile generated to facilitate interpretation and definition of genotypes.

AFLP was performed following the method described by Gibson *et al* (1997). Aliquots (4 μg) of extracted genomic DNA were digested in a 20 μl reaction volume containing 1 μl 0.1 M spermidine trihydrochloride [Sigma Ltd], 2 μl 10 X React 2 buffer [Invitrogen] and 2 μl restriction endonuclease *Hind*III (10 U/μl) [Invitrogen] at 37 °C for 16 - 18 h. A 5-μl aliquot of approximately 1 μg of digested DNA was then used in a 20 μl ligation reaction containing 0.2 μg of each adapter (ADH1 5'-ACG GTA TGC GAC AG-3' and ADH2 5'-AGC TCT GTC GCA TAC CGT GAG-3') [MWG Biotech Ltd] and 1U T4 DNA ligase [Invitrogen] in single strength ligase buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM dithiorthreitol, 5.0 % (*w/v*) polyethylene glycol-8000). Ligation occurred during incubation (3 h) at room temperature and T4 DNA ligase was inactivated by subsequent incubation at 65 °C for 10 min. Digested ligated DNA was then diluted 1/5 (*v/v*) in distilled water and fragments of DNA to which adapters had been ligated were amplified in a 50-μl PCR reaction containing 5 μl of diluted digested ligated DNA, 200 μM (each) dNTPs [Invitrogen], 2.7 nM primer (HI-A 5' GGT ATG CGA CAG AGC TTA 3') [MWG Biotech], 1.5 mM $MgCl_2$ and 1 U of *Taq* polymerase [Invitrogen] in PCR buffer (20 mM TrisHCl, pH 8.4, 50 mM KCl, 0.2 % (*v/v*) glycerol). The PCR reaction was

incubated for 4 min at a denaturation temperature of 94°C, followed by 33 cycles of denaturation (94°C for 1 min), annealing (60°C for 1 min) and extension (72°C for 2.5 min).

Fragments of amplified DNA were separated by electrophoresis in a 1.5 % (*w/v*) agarose gel at 100 V for 3 h 40 min and stained in ethidium bromide (0.5 μg/ml) [Sigma Ltd]. AFLP profiles generated were considered identical if there were no differences between isolates and similar if they differed by one band.

## 2.22 Sequencing of amplicons

Fragments of DNA amplicons can be sequenced by a modification of the PCR reaction that includes dideoxynucleotide trisphosphates (ddNTPs) along with higher concentrations of normal dNTPs. The ddNTPs lack a 3' hydroxy group, thus strand extension by addition of dNTPs via a phosphodiester bond cannot occur. During synthesis of the complementary DNA strand, random incorporation of a specific ddNTP in place of a normal dNTP results in chain termination. At the end of the sequencing reaction, numerous fragments of different lengths exists, each terminated by the incorporation of a specific dideoxy base. By labelling each ddNTP with a different fluorescent dye, the terminating bases of each fragment can be distinguished. Separation of labelled fragments according to fragment length and sequential monitoring of the fluorescent dye on each fragment enables the DNA sequence to be determined. As the probability of generating a long fragment without random incorporation of a ddNTP is low, compared with the frequency of generation of short fragments, the intensity of fluorescent signal and quality of sequence decreases as fragment size increases, limiting the length of fragment that can be sequenced in a

single PCR reaction. Larger genes are often sequenced by generating multiple overlapping amplicons.

### 2.22.1 Amplicon purification

Unincorporated primers, dNTPs and $MgCl_2$ can interfere with the sequencing PCR reaction, so amplicons to be used as a template for sequencing reaction were purified.

Amplicons sequenced earlier in this study (16S rRNA, *vacA*, *cagA* and *rdxA* genes) were purified using a commercial kit (Wizard® PCR preps DNA Purification System) according to the manufacturer's instructions [Promega UK Ltd]. Briefly, amplicon (50 – 100 µl) was bound to PCR Preps DNA Purification Resin in the presence of GuSCN-containing buffer, this was transferred to a 5-ml sterile syringe and pushed into a Wizard® Minicolumn. Amplicon/resin complexes were washed once by gentle pushing of 80 % (*v/v*) isopropanol through the Minicolumn using the same syringe and the resin dried by brief centrifugation of the Minicolumn (10 000 rpm for 2 min). Amplicon was eluted by addition of 50 µl sterile distilled water, followed by centrifugation of the Minicolumn (10 000 rpm for 20 s).

Amplicons that were sequenced later in the study (*frxA* and *ahpC*) were separated from unincorporated reagents by vacuum filtration (635 Torr) in MultiScreen®-PCR plates [Millipore Ltd] for 10 min and then resuspended by 15 min agitation in 50 µl molecular grade water [Promega UK Ltd] as directed by the manufacturer's protocol [Millipore Ltd].

### 2.22.2 Quantification of sequencing template

Low DNA Mass Ladder [Invitrogen] comprises an equimolar mixture of six DNA fragments of known concentration, ranging in size from 100 bp – 2000 bp.

Comparison of band intensities of unknown DNA with a standard band of this ladder, approximately equivalent in size, allows quantification of unknown samples. Aliquots (1 µl and 0.5 µl) of purified amplicon and 2 µl and 1 µl Low DNA Mass Ladder I were electrophoresed in a 1.0 % (*w/v*) gel at 135 V for 2.5 h and stained in ethidium bromide (0.5 µg/ml). Amplicon concentration was calculated by visual comparison of band intensities, and amplicon diluted to 100 fmol for sequencing.

### 2.22.3 Sequencing reaction

Briefly, 27/50 *rdxA* amplicons, 10/37 *cagA* PCR products as well as 8/15 amplicons from bladder biopsies were sequenced commercially [MWG Biotech Ltd and Cytomyx, Cambridge, UK]. All remaining amplicons, including all *frxA* and *ahpC* genes were sequenced in-house.

Amplicons were sequenced following a modification of the manufacturer's protocol for the CEQ Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit [Beckman Coulter Inc, High Wycombe, Buckinghamshire, UK]. All sequences were determined in both forward and reverse directions in two separate reactions. Briefly sequencing fragments were generated in a 20 µl reaction containing 100 fmol purified amplicon, 3.2 pmol of the appropriate primer and 8 µl DTCS Quick Start master mix (containing sequencing reaction buffer, dNTPs, dye labelled ddUTP, ddGTP, ddCTP and ddATP and polymerase enzyme) by 40 thermal cycles [DNA engine MJ Research] at 96 °C for 20 s, 46 °C for 35 s and 60 °C for 4 min.

### 2.22.4 Purification of sequencing fragments

Sequencing reactions were stopped by the addition of 5 µl stop solution - 1.5 M sodium acetate [Sigma Ltd], 50 mM EDTA [Sigma Ltd] and 20 µg glycogen [Beckman Coulter Inc]. Fragments were purified in CEQ2000 sample plates

[Beckman Coulter Inc] following the manufacturer's instructions. Briefly, fragments were precipitated in ethanol 95 % (*v/v*) [BDH] at –20 °C for 10 min, centrifuged at 4 °C (14 000 rpm for 30 min) and pellets washed twice with cold ethanol 70 % (*v/v*), dried and resuspended in 40 µl Sample Loading Solution [Beckman Coulter Inc]. Reactions were covered with one drop of mineral oil [Beckman Coulter Inc], to prevent degradation of fluorescent dyes. Sequencing fragments labelled with fluorescent terminator dyes were separated and analysed by gel electrophoresis in commercial polyacrylamide gel [Beckman Coulter Inc] in an automated sequencer CEQ2000 [Beckman Coulter Inc].

### 2.22.5 Sequence analysis

Sequences obtained in the forwards direction were aligned with the reverse complemented matched sequence determined in the reverse reaction in GeneBase version 1 [Applied Maths] to identify any sequence mismatches that suggested inaccurate or poor quality data. Sequence chromatograms of both forward and reverse reactions were then examined in Chromas version 1.42 [Griffith University, Brisbane, Australia], to correct any discrepant bases identified. Corrected sequences were then stored in GeneBase version 1 for further analysis of nucleic acid and translation to amino acid sequences.

### 2.22.6 BLASTn analysis of sequences

BLASTn (Basic Local Alignment Search Tool nucleotide) is a program that allows comparison of sequences with all those available in public databases. The matches identified by BLAST with known sequences provides the basis for identification of unknown sequences.

Amplicons generated from bladder biopsies were identified by BLASTn analysis of determined sequences (http://www.ncbi.nlm.nih.gov/BLAST). Additionally, potential specificity of all primer sequences designed in the course of this study and of selected published primers was assessed by BLASTn analysis.

### 2.23 Detection of *H. pylori* antigens from stool specimens

*H. pylori* specific antigen can be detected in stool specimens by an Enzyme Linked Immunosorbent Assay (ELISA) method. Two commercial ELISA kits were examined in this study, the Premier Platinum HpSA kit [Meridian Diagnostics, USA] and the Amplified IDEIA HpStAR kit [DakoCytomation, Cambridgeshire, UK]. The principle of both kits is similar and is summarised in Figure 2.8. Briefly, diluted stool specimens are added to wells coated in specific anti-*H. pylori*-antigen antibody. If present in the stool, *H. pylori* antigen will be captured and can be detected by anti-*H. pylori* antibody conjugate labelled with the marker enzyme horseradish peroxidase. If a specimen is *H. pylori*-positive, the antigen-antibody conjugate complex will remain bound in the wells following repeated washing steps. This can be detected by the addition of chromogenic substrate, trimethylbenzidine, that will be hydrolysed by the bound marker enzyme to produce a colour change from colourless to blue (subsequently changes to yellow on addition of sulphuric acid to stop the reaction). This colour change can be recorded visually or spectrophotometrically. Stools specimens that had been collected originally for PCR-based analyses also were examined for specific antigen by the two commercial kits.

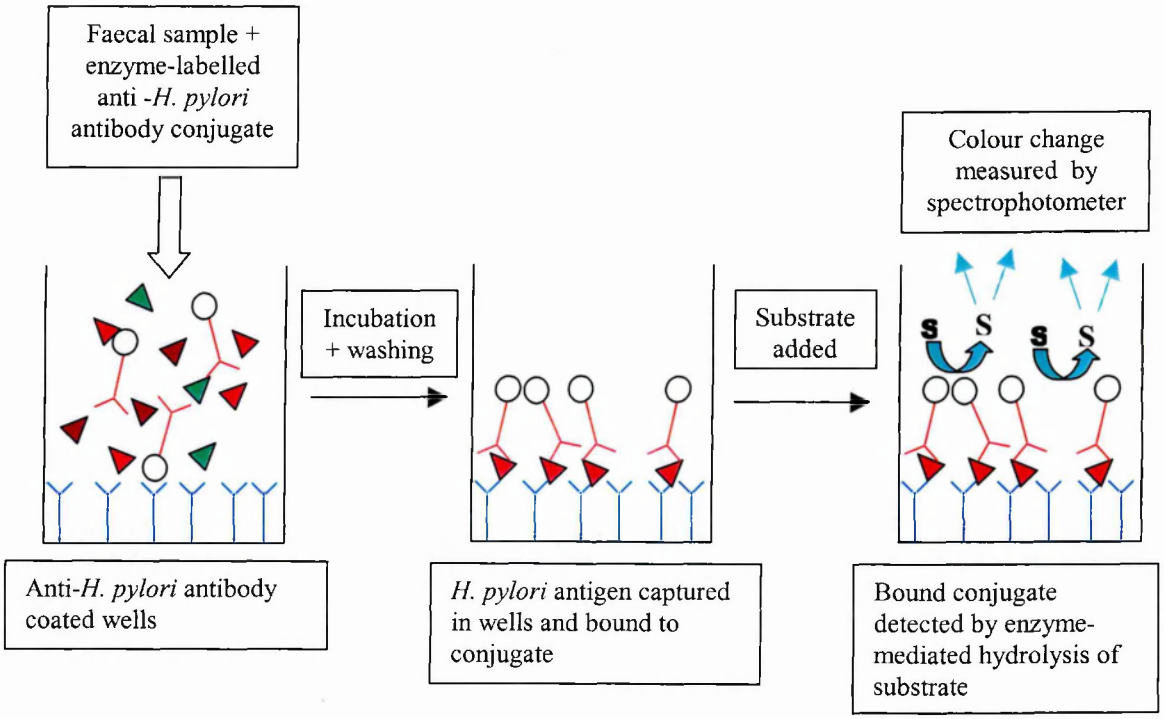



**Figure 2.8: Schematic representation of the detection of *H. pylori* specific antigen in stools by Enzyme-Linked Immunosorbent Assay (ELISA)**

### 2.23.1 Detection of *H. pylori* stool antigen by Premier Platinum HpSA kit

The HpSA kit captures *H. pylori* antigen in wells coated with specific polyclonal antibodies. These antigens were detected from stool samples following the manufacturer's protocols [Meridian Diagnostics]. Briefly, approximately 0.1 g stool was emulsified in 200 μl sample diluent and mixed by vortexing. Diluted stool (50 μl) was added to the antibody-coated well, along with one drop of the kit antibody conjugate. Plates were incubated at room temperature for 1 h and then manually washed 5 times with the buffer provided. Bound antigen-antibody complexes were detected by incubation in the dark with enzyme substrate at room temperature for 10

min, after which time stop solution was added and spectrophotometric absorbances measured at 450 and 630 nm, within 30 min, by a Labsystems Multiskan RC version 6.0 [Labsystems Oy, Helsinki, Finland]. Samples were defined as either negative, equivocal or positive on the basis of $OD_{450/630}$ readings of <0.100, ≥0.100 and <0.120, and ≥0.120, respectively.

### 2.23.2 Detection of *H. pylori* stool antigen by Amplified IDEIA HpStAR kit

The Amplified IDEIA HpStAR kit differs from the HpSA kit in that wells are coated with specific monoclonal antibodies. The procedure stated by the manufacturers [Dako Cytomation] is similar to that described above (2.23.1). Stool was emulsified in 500 µl sample diluent then, following vortexing, centrifuged (5000 rpm for 5 min) and 50 µl supernatant added to antibody-coated wells, along with 50 µl enzyme conjugate. Following incubation (ambient temperature for 1 h), wells were washed using a Well Wash 4 automated plate washer [Denley] and 100 µl substrate added. The reaction was stopped after incubation at ambient temperature for 10 min in the dark by the addition of 100 µl stop solution and results recorded as described above. Samples were defined as either negative or positive on the basis of $OD_{450/630}$ readings of < 0.150 and ≥0.150, respectively.

### 2.23.3 Detection of *H. pylori* stool antigen by ImmunoCard STAT! HpSA kit

In addition to ELISA, a rapid immunoassay was evaluated, that identified *H. pylori*-positive patients by a lateral flow chromatography-based method capturing specific antigen from stools via a specific monoclonal antibody immobilised in a test cassette. Tests were performed according to the manufacturer's instructions [Meridian Diagnostics]. Approximately 5 – 6 mm stool was added to vials containing 1 ml sample diluent and emulsified by vortexing for 15 s. The tip of the vial was broken

off and four drops of stool suspension added to the sample port of the test cassette. The test was read after exactly 5 min incubation at ambient temperature. Tests were interpreted as negative if there was a blue line in the control (C) window only and positive if there was any evidence of an additional pink line in the test (T) window.

## 2.24 Calculation of test sensitivity and specificity

Specificity and sensitivity of tests were calculated using the following formulae (www.musc.edu/dc/icrebm/sensitivity):

$$\% \text{ Sensitivity} = \frac{TP}{TP + FN} \times \frac{100}{1}$$

$$\% \text{ Specificity} = \frac{TN}{TN + FP} \times \frac{100}{1}$$

Where TP = True Positives, TN = True Negatives, FP = False Positives, FN = False Negatives.

## 2.25 Statistical analyses.

All statistical analyses were performed using GraphPad InStat, version 3.05 [GraphPad, San Diego, USA]. The following tests were applied to data sets.

### 2.25.1 Means, standard deviations and 95 % confidence intervals

The mean $OD_{450/630}$ values generated by each stool antigen kits (2.23.1 and 2.23.2) were calculated to compare the relative performances of each kit and also the performance of these in relation to that of the ImmunoCard STAT! test (2.23.3).

The mean of the crossover threshold (CT) values (the point where amplicon generation enters the exponential phase) for LightCycler TPM assays CagMotA and CagMotB (2.20.6) were calculated to interpret performance of these when applied to gastric biopsies.

### 2.25.2 Unpaired *t* test

Mean values generated above (2.25.1) were compared for significant differences by the unpaired *t* test. This is an appropriate test for comparison of two sets of independent quantitative data that do not differ significantly and have similar standard deviations (Swinscow 1996b;Swinscow 1996c). A p value of less than 0.05 indicated significant differences.

### 2.25.3 Fisher's exact test

Potential associations between putative virulence factors (*vacA* genotype and *cagA* TPMs) and disease outcome and PCR positivity and interstitial cystitis were analysed by Fisher's exact test. This test is appropriate for such data where both the input variable (e.g. virulence factor) and output variable (e.g. disease outcome) are nominal, and the study population size is small (Swinscow 1996a;Swinscow 1996c). A p value of less than 0.05 indicated significant differences.

# Chapter 3: Evaluation of the effects of different conditions for transport of gastric biopsies on PCR-based detection of *H. pylori*.

## 3.1 Background

A prerequisite of any diagnostic method is that it should offer high sensitivity and specificity. The performance of a PCR-based detection assay can be affected by various factors, including the nature and quality of a clinical specimen, the method of DNA extraction, the primers used and the conditions of the PCR. To ensure that PCR-based detection of *H. pylori* from clinical specimens was as sensitive and specific as possible, these factors required evaluation, to optimise methodologies for all further investigations.

Previous studies have demonstrated that the transport conditions of gastric biopsies significantly affect success of culture of *H. pylori* (Han *et al.* 1995;Heep *et al.* 1999). Although it is accepted that PCR is capable of detecting bacteria in a variety of metabolic states, including dead or non-cultivable cells, the effects of transport conditions on the outcome of PCR detection for *H. pylori* have not been investigated. As was discussed in section 1.7, numerous *H. pylori*-specific detection assays have been described, but the high inter-assay performance variability could significantly affect the outcome of PCR-based testing of clinical specimens. In addition, the DNA extraction method should be rapid and simple (to allow high throughput processing of clinical samples) while also able to produce a sufficient yield of quality template DNA to allow sensitive and specific detection. Many clinical samples contain substances, such as haem, bile salts and polysaccharides that are inhibitory to the PCR, and an extraction method must also remove as many of these inhibitors as possible. The methods of DNA extraction described for gastric biopsies range from

simple vortexing or boiling of specimens (Bickley *et al.* 1993;Lage *et al.* 1995;Wahlfors *et al.* 1995) to more complex approaches involving phenol chloroform extractions (Chong *et al.* 1996;Lage *et al.* 1995;Li *et al.* 1996;Mapstone *et al.* 1993a). However few studies have compared the effects of different extraction methods on the outcome of PCR testing for helicobacters (Thoreson *et al.* 1995;Wang *et al.* 1993). The aims of the study presented in this chapter were:

1) To establish a method of DNA extraction from gastric biopsies that was rapid but that could provide DNA template containing minimal levels of PCR-inhibitors.
2) To determine the optimal conditions for transport of clinical samples for sensitive and specific detection of *H. pylori*.
3) To compare the performances of three assays targeting different areas of the *H. pylori* genome, namely, *glmM*, 16S rRNA and *vacA* genes, in order to select the best assay(s) for subsequent analyses.

## 3.2 Results

### 3.2.1 Comparison of extraction methods

Ten gastric biopsies that were culture-positive for *H. pylori* were homogenised in sterile saline and divided into two aliquots. One aliquot was extracted by the boiling method (2.11.1) while the other was processed by the digestion method (2.11.2.1).

#### 3.2.1.1 Internal control PCR to confirm removal of inhibitory substances

A PCR assay that specifically amplifies a fragment of a human cytochrome oxidase gene (2.15.1) was applied to all 20 biopsy DNA extracts as an internal control to evaluate the ability of each extraction method tested to remove PCR-inhibitory substances. Amplicons of the appropriate size (823 bp) were generated in all DNA preparations from gastric biopsies, regardless of extraction method.

### 3.2.1.2 PCR-based evaluation of DNA recovery

The *glmM* gene-specific PCR assay generated amplicons from 9/10 biopsy DNA preparations, regardless of the extraction method used. However, the intensity of the amplified fragments was generally slightly higher for some of the DNAs extracted by the digestion method, as illustrated in Figure 3.1.

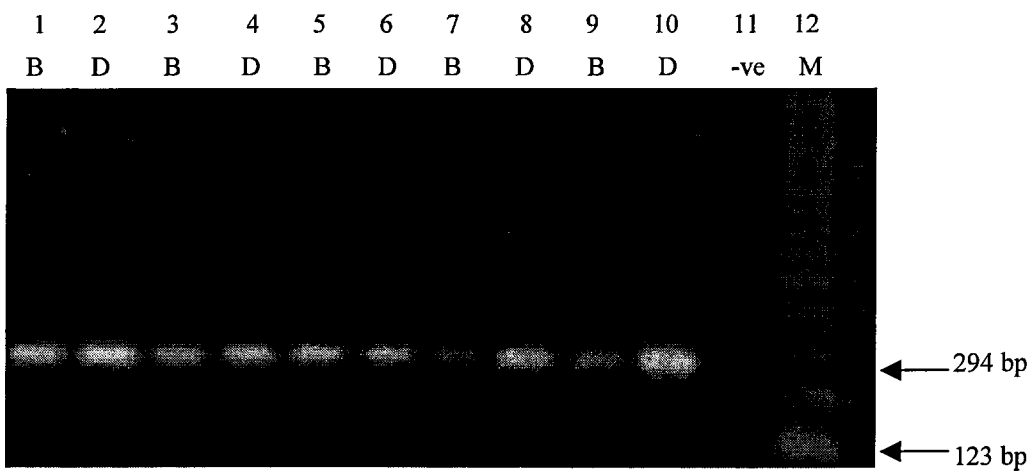



**Figure 3.1 Examples of *H. pylori* detection by *glmM* amplification using DNA extracted from human gastric biopsies by boiling (B) or digestion (D) methods.** Lane 1-10, DNA from human gastric biopsies extracted by B or by D method; lane 11, negative control; lane 12, 123 bp molecular weight marker (M).

### 3.2.2 Comparison of effects of transport methods on PCR

The effect of six different combinations of transport conditions on the performance of three PCR assays was evaluated. The biopsies studied were collected from dyspeptic

patients as part of a routine endoscopic examination. Seven biopsy groups were defined according to source, method of determination of *H. pylori*-status and conditions of storage and transport. The *H. pylori* status of biopsies was determined by culture and histology in Groups 1 – 5, obtained from Chelmsford PHL. All biopsies in Groups 1 and 5 were *H. pylori*-positive, while for Groups 2, 3 and 4, biopsies were obtained prospectively and 11/41, 16/70 and 17/121, respectively, were *H. pylori*-positive. Group 6 (n = 30) comprised a miscellaneous collection of biopsies submitted to the HRU for routine culture. Additional biopsies had undergone histological examination at the respective hospital. The Group 6 biopsies had been received from the Royal Free Hospital, London (n = 7), the University College Hospital, London (n = 4) and King's College Hospital, London (n = 19); 19/30 of these were *H. pylori*-positive. Group 7 was obtained from North Middlesex University Hospital, where demonstration of rapid urease activity (CLO test) confirmed the presence of *H. pylori* in 60/72 biopsies.

Transport conditions for each biopsy group, summarised in Table 3.1, were as follows:

Group 1: Specimens had been frozen in physiological saline at the primary laboratory at the time of culture and all cultures and matched biopsies forwarded to the HRU at room temperature where they were stored (-20 °C) for further processing.

Group 2: Biopsies were not frozen at the primary laboratory but both culture-positive and negative specimens were forwarded to the HRU as described for Group 1.

Group 3: Transport conditions for biopsies were identical to those of Group 2, except that Dent's transport medium was used instead of saline.

For both Groups 2 and 3, there was a 3-4 day delay between the time that the biopsy was taken by endoscopy and receipt of specimen in the HRU.

Group 4: Specimens were frozen in Dent's medium on the day of endoscopy and were maintained at –20 °C for storage, including transport to the HRU until DNA extraction.

Group 5: Biopsies, collected for a previous study (Peters *et al.* 1997), had been transported and maintained under identical conditions as for Group 4.

Group 6: Biopsies were transported in Dent's medium to the HRU for routine culture on the same day as endoscopy and were stored (–20 °C) for molecular analyses.

Group 7: Biopsies were tested for rapid urease activity (CLO test) at North Middlesex University Hospital and were transported each week in batches to the HRU in the original CLO test media.

All gastric biopsies were tested for *H. pylori* DNA by three assays targeting *glmM*, 16S rRNA and *vacA* genes. The results of these analyses are presented in section 3.2.4.

Selected biopsies from these defined groups were used also in additional studies presented in Chapters 4 – 7.

**Table 3.1: Summary of storage and transport conditions of human gastric biopsies analysed by PCR.**

| Transport Conditions | Biopsy Group (number of samples) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **1** (14) | **2** (41) | **3** (70) | **4** (121) | **5*** (39) | **6** (30) | **7** (72) |
| Initial storage Temp.(°C) | -20 | 4 | 4 | -20 | -20 | 4 | NR† |
| Transit Temp. (°C) | ambient | ambient | ambient | <0 | <0 | ambient | ambient |
| Transport media | Saline | Saline | Dent's | Dent's | Dent's | Dent's | CLO test |
| Mean transit time to HRU | NR | 110 h | 93 h | 2 h | 2 h | 3-4 h | >72 h |

*Archival collection

† Not Recorded

### 3.2.3 Development and optimisation of novel *H. pylori*-specific PCR assay HpVac

Alignment of 30 complete *vacA* sequences retrieved from GenBank (Appendix B.1) identified conserved regions at the 3' end suitable for design of primers that would amplify all *H. pylori* strains. BLASTn analysis of candidate primers demonstrated that primers vac3624F and vac3853R were *H. pylori*-specific, and no significant homologies with human DNA sequences were found. Initial application of the resultant PCR assay (HpVac) to 30 clinical isolates of *H. pylori* generated specific (230 bp) product in all cases. Analysis of five *H. pylori*-positive and five *H. pylori*-negative biopsies selected from Groups 4 and 5 generated specific amplicon in all positive biopsies. However, larger non-specific bands were also observed in 7/10 biopsies and although raising the annealing temperature to 53 °C decreased the intensity of these bands it did not eliminate non-specific amplification in 3/10

biopsies. Further attempts to optimise $MgCl_2$ and primer concentration failed to reduce the generation of non-specific bands. Examples of amplicons generated from three patients in Group 7 are provided in Figure 3.2, to illustrate the quality of results obtained.

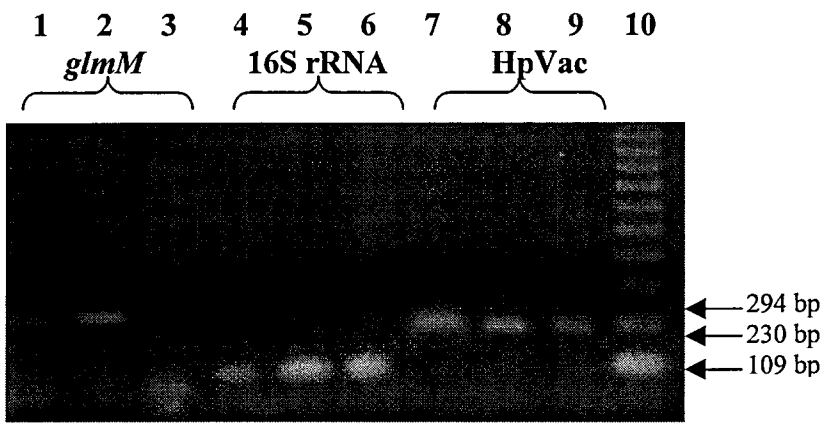



**Figure 3.2: Examples of *H. pylori* detection in Group 7 gastric biopsies by three different *H. pylori*-specific PCR assays targeting *glmM*, 16S rRNA and *vacA* genes.** Patient A, lanes 1, 4 & 7; Patient B, lanes 2, 5 & 8; Patient C, lanes 3,6 & 9. 123 bp ladder, lane 10. All biopsies were positive for all PCR assays, except for a false negative result generated by the *glmM* assay for patient C (lane 3).

### 3.2.4 Determination of sensitivities and specificities of *H. pylori*-specific PCR detection assays

The relative sensitivity of each PCR assay was determined by testing of serial decimal dilutions of *H. pylori* (NCTC 11637) DNA ranging from 100 ng – 10 fg. The detection limit was 100 fg for the HpVac assay, and 1000 fg for both the 16S rRNA and the *glmM* assays.

The sensitivity and specificity of the three assays when applied to gastric biopsies was determined for each by the formulae described in section 2.24. A biopsy that was positive by culture, histology or CLO-test but was PCR-negative was considered falsely-negative while amplicon generation in a biopsy that had been negative by culture, histology or CLO-test was classified as falsely positive. As all biopsies in Groups 1 and 5 were *H. pylori*-positive and the proportion of *H. pylori*-negative biopsies in Group 6 was low, specificities could only be determined in Groups 2, 3, 4 and 7. Calculated sensitivities and specificities for individual PCR assays when applied to each gastric biopsy group are presented in Table 3.2.

**Table 3.2: Effect of transport conditions on sensitivity and specificity of *H. pylori*-specific PCR assays targeting *glmM*, 16S rDNA and *vacA***

| PCR Assay | | Biopsy Group* (n) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 (14) | 2 (41) | 3 (70) | 4 (121) | 5 (39) | 6 (30) | 7 (72) |
| ***glmM*** | **Sensitivity (%)** | 28.6 | 18.2 | 56.3 | 82.3 | 92.3 | 84.2 | 55.0 |
| | **Specificity (%)** | na† | 100.0 | 98.1 | 99.0 | na | na | 83.3 |
| **16S rDNA** | **Sensitivity (%)** | 57.0 | 72.7 | 87.5 | 82.3 | 92.3 | 84.2 | 76.7 |
| | **Specificity (%)** | na | 100.0 | 92.5 | 98.1 | na | na | 83.3 |
| **HpVac** | **Sensitivity (%)** | 57.0 | 81.8 | 93.8 | 88.2 | 92.3 | 89.5 | 78.3 |
| | **Specificity (%)** | na | 100.0 | 98.1 | 99.0 | na | na | 83.3 |

*Biopsies grouped according to transport conditions (Table 3.1): n = number of samples.

†Not applicable as all biopsies were culture-positive.

## 3.3 Discussion

### 3.3.1 Evaluation of DNA extraction methods for gastric biopsies

Many clinical samples contain substances that inhibit the action of *Taq* polymerase in the PCR reaction (Lantz *et al.* 2000;Wilson 1997), with complex specimens such as blood, saliva and stools being particularly problematic. PCR-based amplification of *H. pylori* DNA direct from gastric biopsies is widely reported, with numerous different DNA extraction methods described. The approaches described range from simple vortexing and/or boiling (Clayton *et al.* 1992;Thoreson *et al.* 1999;Wahlfors *et al.* 1995) or digestion in lysis buffer (Marais *et al.* 1999;Namavar *et al.* 1995;Weiss *et al.* 1994) to more complex multiple-step protocols involving phenol chloroform extraction (Chong *et al.* 1996;Engstrand *et al.* 1992;Hammar *et al.* 1992;Li *et al.* 1996) or commercial extraction kits (Lu *et al.* 1999). Although essential for some specimen types containing high levels of inhibitors, complex DNA extraction protocols are undesirable as they lengthen the overall processing time and increase the risk of introducing DNA from exogenous sources. A previous study using internal control PCR demonstrated that 42 % of gastric biopsies contained inhibitory substances (Thoreson *et al.* 1999), while another study suggested that greater DNA yield was obtained from biopsies extracted by phenol chloroform-based methods (Wang *et al.* 1993). A priority of this study was to establish a method of DNA extraction that was rapid, simple and effective, to minimise the potential for false-positive PCR results due to DNA contamination or false-negative results due to PCR inhibition.

Two extraction methods described previously (Bickley *et al.* 1993;Marais *et al.* 1999) were evaluated; both of which met these criteria. An internal control PCR targeting the human cytochrome oxidase gene demonstrated that extracts prepared by

either method did not contain significant levels of PCR inhibitors. While the heating steps employed in both methodologies would denature proteins and degrade other labile substances, neither contained steps specifically aimed at removing inhibitory substances. It is likely, therefore, that human gastric biopsies are not a source of substances inhibitory to PCR, even in specimens where blood is clearly visible. Of the two methods tested, the boiling method of Bickley *et al* (1993) was simpler and approximately 50 minutes faster than the digestion method described by Marais *et al* (1999). Although both methods were equally sensitive in detecting the *glmM* gene of *H. pylori*, visual comparison of intensity of PCR bands in an agarose gel showed that in some cases the digestion method generated stronger amplicons. As the recovery of specific DNA was apparently less efficient by boiling than by digestion, all subsequent gastric biopsies were extracted by the digestion method.

### 3.3.2 Evaluation of the effects of biopsy transport conditions on *H. pylori* detection by PCR

At the time this study was initiated over 15 different PCR assays targeting various genes had been developed for the detection of *H. pylori*, as described in section 1.7. In the majority of cases these assays were reported to be highly sensitive and specific, however many had not been evaluated critically or employed subsequently by other workers. One such independent study compared the five PCR assays of Ho *et al* (1992), Hammar *et al* (1992), Valentine *et al* (1991), Clayton *et al* (1991) and Brisou *et al* (1990), targeting, respectively, 16S rDNA, a 26 kDa species-specific antigen gene, a random sequence, the *ureA* and the *glmM* genes. Analysis of 50 gastric biopsies, 24 of which were culture-positive, demonstrated that the *glmM* assay was the most sensitive and specific (Lu *et al.* 1999). Consequently the *glmM* PCR assay was selected for further evaluation in the initial stages of this study. Additionally,

primers Hp1 and Hp2 described by Ho *et al* (1992) were selected for evaluation as these had been applied successfully in a number of separate studies, albeit in different nested and semi-nested combinations (Chong *et al.* 1996;Mapstone *et al.* 1993a;Nguyen *et al.* 1995;Nilsson *et al.* 1999).

Gastric biopsy specimens were collected prospectively from dyspeptic patients as part of routine endoscopic investigation. The biopsy Groups 1 - 4 were defined on the basis of their chronological collection over a 10-month period and the progression in different transport conditions employed. Analysis of Group 1 demonstrated that sensitivity of *H. pylori* detection was low for both PCR assays. Internal control PCR amplifying human cytochrome oxidase DNA demonstrated that false-negatives were not due to the presence of residual substances inhibitory to the PCR reaction following extraction. It was hypothesised that the initial freeze-thawing cycle may lyse human cells, liberating DNAses that degraded specific target during transit. However, the sensitivity of detection was also low for Group 2 biopsies that had not undergone this process. Some studies have demonstrated that transport or storage of *H. pylori* in saline decreases the recovery rate (van der Hulst *et al.* 1996) and cell stability (Heep *et al.* 1999), thus transport of biopsies in saline may be insufficient to support *H. pylori* survival. Autolysis of bacterial cells would expose the target DNA to bacterial and human DNAses, resulting in decreased sensitivity of PCR-based detection.

Transport of biopsies in Dent's medium instead of physiological saline (Group 3) increased sensitivity of both assays but seven biopsies were still falsely negative by the *glmM* assay. Three false-positives were generated by the assay targeting the 16S rRNA gene only in this group but these were too weak to allow further characterisation by sequencing. Although antimicrobial agents are included in Dent's

to inhibit growth of many contaminants, this richer medium could support the growth of other bacterial species. 16S rRNA genes are universally present in bacteria and cross-reaction with the *H. pylori*-specific 16S rRNA primers would generate falsely positive results. This was most likely for Groups 2 and 3 as there was a considerable delay (between two to four days) between receipt of the biopsy in the primary laboratory for culture and transit to the HRU. The delay would not only allow overgrowth of contaminants in Dent's medium but prolonged incubation at temperatures where DNAse activity could degrade specific DNA would also account for the poor sensitivity of PCR detection in these samples. Further evidence that this may be the most likely explanation for the high rate of false-negative results was provided by the higher sensitivities observed for both assays when applied to stored (-20 °C) biopsies from Groups 4 to 6 that had immediately been frozen on the same day as endoscopy. In contrast, the low sensitivity of detection observed in Group 7 biopsies that had been transported in CLO test medium provided additional evidence of the adverse effects of prolonged storage at ≥4 °C prior to DNA extraction. Additionally, the conditions provided by the CLO test medium might have accelerated cellular and DNA degradation. Culture of these biopsies on arrival in the HRU showed that organisms were no longer viable. Nevertheless, 80.0 % of CLO-test biopsies were positive by at least one PCR assay, demonstrating the power of PCR methodology for detection of non-viable organisms.

Loss of sensitivity of PCR-based detection due to DNA degradation has been described previously for the parasite *Toxoplasma gondii* (James *et al.* 1996). In the case of *H. pylori*, many reports focus on the value of PCR-based detection from gastric biopsies, as described in section 1.7, and yet the problems of specific DNA degradation were not addressed, perhaps because the investigating laboratory was in

close proximity to the endoscopy clinic. The results presented here suggest that the transit time for biopsies transported at room temperature must be short (ideally < 24 hours) or the sensitivity and specificity of PCR detection is severely compromised. However, where rapid transport between laboratories is difficult, for example due to geographical distance, the optimal handling procedure would appear to be immediate freezing of biopsies in Dent's medium and maintenance of freezing until DNA extraction. Other workers demonstrated that sensitivity of culture from gastric biopsies sent to geographically distant locations was highest if specimens were transported in media other than saline at a temperature of <4 °C (Heep *et al.* 1999). This issue is particularly important to a reference unit function, as PCR-based testing of clinical specimens could be offered as a nationwide service in the future to hospitals that do not culture for *H. pylori*. In such situations, transport delays due to geographical distance would be inevitable.

### 3.3.3 Comparison of performances of *H. pylori*-specific 16S rRNA and *glmM* PCR assays

Although specimen quality appears to be a critical factor in PCR-based detection of *H. pylori*, the different performance characteristics of individual assays documented previously (Lu *et al.* 1999) were also re-assessed in this study. Both assays (targeting the 16S rRNA and the *glmM* genes) performed similarly when applied to serially diluted DNA from culture and to biopsies that had been stored at –20 °C (Groups 4 and 5). However sensitivity of *glmM* PCR was lower than that of the 16S rRNA assay in biopsy Groups 1 to 3 and in Group 7. This suggests that for samples where levels of target DNA are low (either due to sample deterioration or an initially low bacterial load) the *glmM* assay is insufficiently sensitive to be applied as a detection method, although it could be considered a useful indicator of sample quality.

Originally this target was thought to be *H. pylori*-specific (Labigne *et al.* 1991) but later was shown to encode phosphoglucosamine mutase (*glmM*) that is present in other bacterial species (De Reuse *et al.* 1997). In spite of the potential for cross-reactivity, no false-positive results were generated in the present study. The 16S rRNA PCR assay was marginally more sensitive in the same sample groups, but specificity was slightly lower. Poor specificity is a potential problem with many PCR assays targeting rRNA genes, as those sequences are ubiquitous. Furthermore, contamination of gastric biopsies with other bacterial species, often from the oral cavity, is not infrequent in routine culture, particularly for patients on acid suppressive therapy. It might be expected that this assay could generate falsely positive results, particularly in specimens where transport to the molecular laboratory is delayed, as was the case in Groups 2 and 3. Specificity problems have been reported previously where product that was considered falsely-positive was amplified from a range of human clinical specimens, including gastric, colonic and liver biopsies, as well as in stools and in human leucocytes (Chong *et al.* 1996).

### 3.3.4 Development and evaluation of a novel *H. pylori*-specific PCR assay

As neither of the two assays discussed above achieved high-level sensitivity and specificity, a novel assay (HpVac) amplifying the *vacA* gene was developed. This target was selected as firstly, it is only found in *H. pylori* and secondly, all strains possess the gene, although not all express functional vacuolating cytotoxin (Cover 1996). Consequently an assay targeting *vacA* has the potential to be highly specific. As discussed in section 1.12.1, some regions of *vacA*, such as the mid (m)-region, are highly diverse. The HpVac assay was designed to target a conserved region based on *in silico* comparisons of *vacA* sequences retrieved from GenBank. Despite extensive optimisation attempts, non-specific bands were amplified in approximately 10 % of

gastric biopsy DNA extracts. These were significantly larger (650 bp and 1.2 kb) than the predicted specific 229-bp amplicon, and therefore erroneous reporting of a false-positive result was unlikely. The origin of these bands was not known but they were generated occasionally in both culture-positive and culture-negative biopsies but not from DNA extracted from pure culture of the infecting strain of *H. pylori*. It was therefore most likely that human DNA had been amplified. Generation of non-specific DNA bands could theoretically reduce the sensitivity of the HpVac assay by competitive co-amplification of specific and non-specific product. However, this was not observed, with strong *H. pylori*-specific amplicons generated regardless of non-specific amplification.

Analysis of serially diluted DNA from culture demonstrated the HpVac assay was more sensitive (X 10) than either the *glmM* or 16S rRNA assays. Likewise, retrospective application of HpVac to biopsy Groups 1 to 7 demonstrated that it was considerably more sensitive than the *glmM* assay in Groups 1 to 3 and Group 7, and marginally more sensitive and specific than the 16S rRNA assay. Occasional discrepancies between *vacA* and 16S rRNA results occurred that may be due to sequence variation at the primer binding sites generating occasional false-negative results. *H. pylori* is a highly heterogeneous species at the genome level with a panmictic population structure (Suerbaum *et al.* 1998), so the potential for primer site mismatch is high. It is therefore unlikely that any single PCR assay will amplify all *H. pylori* strains encountered. For this reason, both 16S rRNA and HpVac assays were used in subsequent studies to maximise sensitivity of detection.

### 3.3.5 PCR-based detection of non-viable organisms

Of all the negative biopsies analysed, four biopsies that had been designated as *H. pylori*-negative were PCR-positive for all three assays. Although these results were

included in calculations as falsely positive, it is more likely that the original conventional diagnostic tests were falsely negative. Only two of 198 biopsies were falsely negative when tested by culture and histology, indicating that this error occurs relatively rarely. In contrast 2/12 CLO test-negative biopsies tested were PCR-positive. The colour of the pH indicator in these two tests had been slightly more orange than the expected yellow of a negative result – thus equivocal results that can be misinterpreted as negative may occur relatively frequently. *H. pylori* infections are diagnosed solely by CLO test in many gastroenterology clinics in the UK and elsewhere (Heep *et al.* 2001). Extension of this study to include larger numbers of CLO test-negative biopsies for PCR analysis would enable the reliability of the CLO test to be assessed. This highlights the power of PCR as *H. pylori* could not have been detected from these two specimens by any other means. PCR could serve as a supplementary method for those CLO test results that are difficult to interpret and this approach would not require any additional specimen collection.

## 3.4 Conclusions

PCR-based detection of *H. pylori* direct from gastric biopsies allows rapid (potentially same-day) diagnosis of infection that is highly specific and relatively sensitive. However conditions of transport and length of time between endoscopy and DNA extraction play a crucial role in PCR assay sensitivity, with some tests (notably *glmM*) more affected than others (16S rRNA and HpVac). An appreciation of the problems of DNA degradation during transport is vital to ensure that molecular tests provide a robust, accurate alternative to traditional diagnostic methods. Additionally, no single PCR assay will detect all strains of the highly heterogeneous *H. pylori* and thus a multiple-assay approach may be the best means of maximising sensitivity. PCR can

be useful for interpreting equivocal CLO-test results and is a powerful tool for the further investigation of clinical specimens. This facility will be explored in subsequent sections.

# Chapter 4: Development of a novel multiplex PCR assay for detection of *H. pylori* and '*H. heilmannii*'-like organisms direct from human gastric biopsies.

## 4.1 Background

The previous chapter focused on developing methods for transporting and processing gastric biopsies for molecular analyses. PCR based detection of *H. pylori* enables same-day diagnosis of infection, however a range of other diagnostic methods are also available. In contrast, as discussed in section 1.13.3, '*H. heilmannii*'-like organisms (HHLOs) remain uncultivable, with rare exceptions (Andersen *et al.* 1996), and diagnosis is primarily reliant on histology. While one PCR-based detection assay is described for '*H. heilmannii*' detection in domestic cat biopsies (Neiger *et al.* 1998), PCR-based detection of human infection is reported for a single patient only (Dieterich *et al.* 1998). As microscopy-based detection methods can be relatively insensitive when bacterial levels are low (Holck *et al.* 1997;Megraud 1995) the incidence of HHLO infection may be under-reported in England and elsewhere.

The aims of the study presented in this chapter were:

1) To develop a novel PCR-based assay to detect all known HHLOs direct from human gastric biopsy to facilitate surveillance of such infections in England.
2) To combine this test with a PCR detection assay for *H. pylori* to enable rapid detection of both HHLO and *H. pylori* infections of the human gastric mucosa.
3) To apply this novel assay to a collection of biopsies that were *H. pylori* positive by either culture or CLO test to determine possible co-infection with HHLOs.

4) To apply this assay to biopsies that were considered *H. pylori* positive by CLO test to investigate the possibility that misdiagnosis of *H. pylori* infection may occur as a result of HHLO urease activity.

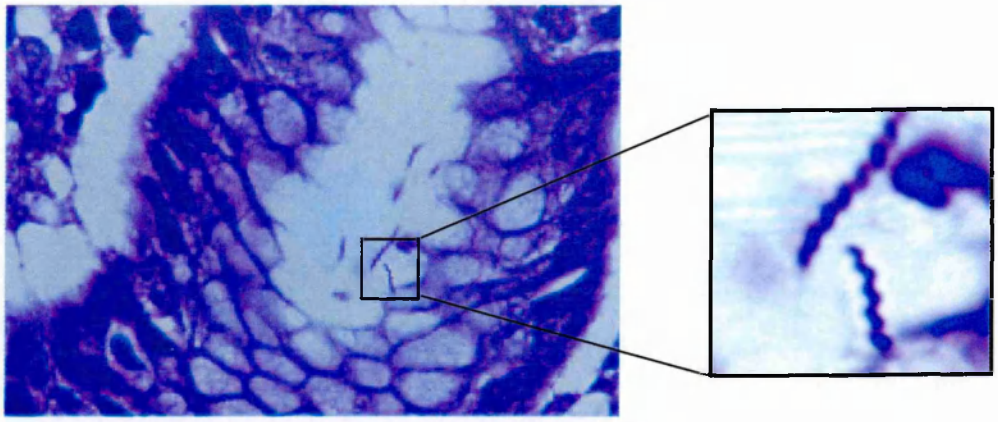
## 4.2 Results

### 4.2.1 Biopsy specimens

Gastric biopsies analysed in this study comprised 230 human gastric biopsies selected from biopsy Groups 4 (n = 116), 5 (n = 39), 6 (n = 15) and 7 (n = 60), defined in Chapter 3, and an additional 15 feline gastric biopsies collected from domestic cats undergoing routine veterinary investigation.

### 4.2.2 Development and evaluation of an HHLO specific assay

Primers that amplified a specific fragment of the 16S rRNA gene of HHLOs were developed as described in section 2.16.1.4. Application of the resultant HHLO-specific assay, termed HHLO-16, to 22 strain DNAs representing 12 other species of *Helicobacter* (Appendix A.1) demonstrated that the assay did not amplify the 112-bp 16S rDNA fragment from *H. pylori* or from any species of *Helicobacter* other than *H. felis*. The latter result was consistent with the findings of a BLASTn search that indicated the HHLO-16 assay would theoretically detect members of the *H. felis*, *H. bizzozeronii*, *H. salomonis* group (= *'H. heilmannii'* type 2). However, DNA from the latter two species was not available for testing. Application of the HHLO-16 assay to a human gastric biopsy (from Group 6) for which there was histological evidence of an HHLO infection (Figure 4.1) generated the expected 112-bp amplicon (Figure 4.2).



X 100 magnification

**Figure 4.1: Histological evidence of HHLO infection in a human antral gastric biopsy that was PCR-positive by the HHLO-16 assay, but not by the *ureB* assay** (slide reproduced by kind permission of Drs S. and J. Dobbs and Professor A. Price).

Sensitivity of the HHLO-16 assay was assessed by testing 15 gastric biopsies from domestic cats, where the incidence of infection with HHLOs was expected to be high according to reported prevalence rates (Norris *et al.* 1999). The HHLO-16 assay generated HHLO-specific amplicons in 13/15 feline biopsies, two of which were confirmed as containing spiral organisms by Gram stain (Table 4.1). Further analyses of the cat biopsies (as described in sections 2.16.2.2 and 2.16.2.3) by the two *ureB* PCR assays of Neiger *et al.* (1998), that were either *'H. heilmannii'* or *H. felis*-specific, demonstrated that all 13 of these were positive for '*H. heilmannii*' (= type 2), as indicated by visualisation of a 580-bp band, while a 1150-bp band was only observed in one biopsy that was thus also positive for *H. felis* (Table 4.1). Spiral organisms were observed in that particular biopsy by Gram staining and microscopy. The relative performances of the HHLO-16 assay and the two *ureB* assays in terms of

sensitivity and specificity, and examples of the quality of PCR products generated are presented in Table 4.1 and Figure 4.2, respectively.

**Table 4.1: Comparison of the performance of the novel HHLO-16 PCR assay, applied to human and to feline gastric biopsies with *'H. heilmannii'* or *H. felis* specific assays targeting *ureB*.**

| Gastric biopsy (n) | PCR Assay | | | | | |
|---|---|---|---|---|---|---|
| | HHLO-16 | | *'H. heilmannii'** | | *H. felis* * | |
| | +(†) | - | +(†) | - | +(†) | - |
| Human (131) | 3(1) | 128 | 1(0) | 130 | na‡ | na |
| Feline (15) | 13(2) | 2 | 13(2) | 2 | 1(1) | 14 |

*Published assays targeting the urease B subunit (*ureB*) (Neiger *et al.* 1998).
†Number of results confirmed as positive by microscopy (histology or Gram stain).
‡*H. felis* assay was not applied to human gastric biopsies.

### 4.2.3 Determination of prevalence of infection with HHLOs in humans.

Application of the HHLO-16 assay to 131 biopsies from Groups 4 and 6 generated specific amplicons of 16S rDNA in 3/131 DNA extracts (Table 4.1), indicating a prevalence rate of 2.3 % in a sample of dyspeptic patients from South East England.

### 4.2.4 Optimisation of PCR-based multiplex assay (HpHh) for simultaneous detection of *H. pylori* and HHLOs.

The HHLO-16 assay was combined in a multiplex format with the *H. pylori*-specific assay targeting *vacA*, described in the previous chapter. However, analysis of a DNA preparation from a HHLO-positive biopsy seeded with 100 ng *H. pylori* DNA showed that only the HHLO 16S rDNA fragment was amplified. A modified version of this assay that used a different reverse primer (vac4041) in combination with the original forward primer (vac3624) successfully allowed co-amplification of both targets, *vacA* and 16S rDNA (Figure 4.2). Initial evaluation of the multiplex system on fifteen selected feline and human gastric biopsies demonstrated that specific *vacA* amplicons were generated in all five *H. pylori* culture-positive biopsies tested. Specific 16S rDNA amplicons were generated also in all four DNA extracts from HHLO-positive biopsies of both human (2) and feline (2) origin (Figure 4.2). However, a band of similar size to that of *H. pylori*-specific product (417 bp) was generated in a *H. pylori*-negative biopsy. This proved to be non-specific amplification and was eliminated by raising the annealing temperature during thermal cycling from 53 °C to 55 °C. However, this lowered the sensitivity of amplification of 16S rDNA fragments from biopsies that had previously been HHLO PCR-positive, with the generation of three falsely negative results. Final optimisation of the multiplex assay to include unequal concentrations of primers that amplified either *vacA* or 16S rRNA and slight lowering of the annealing temperature to 54 °C, successfully overcame these problems and this method (section 2.16.4) was subsequently applied as described below.

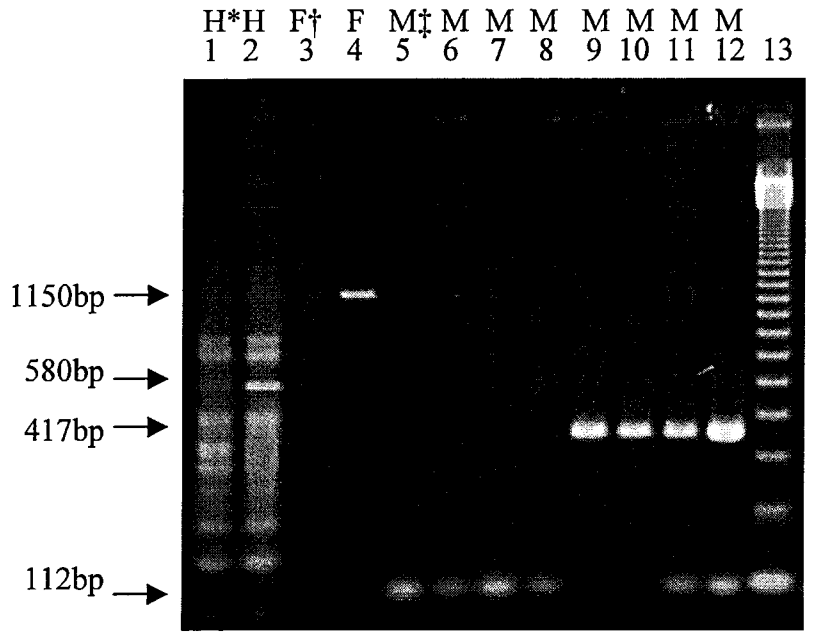



**Figure 4.2: Examples of PCR products generated by '*H. heilmannii*' specific (H*) and by *H. felis* specific (F†) *ureB* assays (Neiger *et al*, 1996) and by multiplex assay HpHh (M‡) amplifying 16S rDNA and *vacA* fragments of HHLOs and *H. pylori* respectively.**

Human biopsy: negative for '*H. heilmannii*' (*ureB*) (lane 1), and *H. felis* (*ureB*) (lane 3) but positive by histology and by assay HpHh (lane 5). Feline biopsy positive by both *ureB* assays (lanes 2 & 4) and by HpHh (lane 6); lanes 7 & 8, HHLO-positive human biopsies; lanes 9 & 10, *H. pylori*-positive human biopsies; lanes 11 & 12, HHLO-positive biopsies spiked with *H. pylori* DNA; lane 13, 123-bp size marker.

### 4.2.5 Evaluation of *H. pylori* and HHLO-specific multiplex PCR assay

The sensitivity of the optimised multiplex system, designated HpHh, was tested by repeat analysis of samples for which the PCR status had already been determined by

uniplex PCR assays. Sensitivities and specificities of both assays when applied to all 230 human and 15 feline biopsies, either individually or in the combined multiplex format are presented in Table 4.2.

**Table 4.2: Comparison of sensitivities of the *vacA* and HHLO-16 assays when applied individually and combined in a multiplex assay HpHh.**

| Gastric Biopsies | | PCR assays | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *H. pylori* (*vacA*) | | | | HHLO-16 (16S rRNA) | | | |
| Origin (n) | Status* | + | - | % Sensitivity | | + | - | % Sensitivity† | |
| | | | | Uniplex | Multiplex | | | Uniplex | Multiplex |
| Cat (15) | C/M + | 0 | 0 | na | na | 13/12‡ | 0/1‡ | 100.0 | 92.3 |
| | C/M - | 0 | 15 | | | 0 | 2 | | |
| Human (170) | C/H + | 59 | 4 | 93.6 | 93.6 | 0 | 63 | 100.0 | 100.0 |
| | C/H - | 0 | 107 | | | 3 | 104 | | |
| Human (60) | U + | 48 | 12 | 80.0 | 80.0 | 0 | 60 | na | na |

**H. pylori* status determined by culture (C), Gram stain microscopy (M), histology (H) or rapid urease (CLO) test (U).

† Sensitivity of HHLO-16 assay calculated on the basis that no PCR-negative biopsies were histology-positive.

‡Results generated by uniplex/multiplex format.

### 4.2.6 Investigation of the frequency of dual *H. pylori*/HHLO co-infections.

The HpHh multiplex PCR assay was applied to 123 human gastric biopsies that were positive for *H. pylori*, either by culture and/or histology (n = 63) or by CLO test (n = 60). Specific targets *vacA* (*H. pylori*) and 16S rDNA (HHLOs) were amplified in 59/63 and 0/63 culture and/or histology-positive biopsies and in 48/60 and 0/60 of CLO test biopsies, respectively (Table 4.2).

## 4.3 Discussion

### 4.3.1 Current methods for identification of HHLO infections

Although the incidence of HHLO infections is high in animals like cats, dogs and pigs, colonisation of the human gastric mucosa is less common (Andersen *et al.* 1996;Dieterich *et al.* 1998;Hilzenrat *et al.* 1995;Holck *et al.* 1997;Jhala *et al.* 1999;Mention *et al.* 1999). As discussed in section 1.13.3, PCR-based studies have identified *'H. heilmannii'* in both human (Chen *et al.* 1997;Morgner *et al.* 2000) and animal (Norris *et al.* 1999) gastric biopsies by amplification of *Helicobacter* genus-specific fragments of 16S rDNA followed by sequence analysis. One species-specific PCR assay, targeting the *ureB* gene, has been described (Neiger *et al.* 1998) that has been applied predominantly for investigation of *'H. heilmannii'* infections in animals and only one human case (Dieterich *et al.* 1998). The development of validated assays for detection of HHLOs is difficult because reference material is not available. Although culture of an organism thought to be '*H. heilmannii*' has been reported (Andersen *et al.* 1996;Andersen *et al.* 1999), it was subsequently shown to be *H. bizzozeronii* (Jalava *et al.* 2001). Thus diagnosis of human *'H. heilmannii'* infection currently relies primarily on microscopy-based approaches such as histological examination (Debongnie *et al.* 1994;Hilzenrat *et al.* 1995;Mention *et al.* 1999;Svec *et al.* 2000;Trebesius *et al.* 2001). The original case study report of this infection stated that histology slides had to be examined for at least five minutes, otherwise the risk of a false negative result would be high (McNulty *et al.* 1989). It is thus hypothesised that diagnosis by histology could be relatively insensitive and consequently, the incidence of human '*H. heilmannii*' infection could be underestimated in the UK and elsewhere.

### 4.3.2 Development and evaluation of HHLO-16 assay

To test the possibility discussed above, a novel PCR based assay (HHLO-16) was developed to amplify fragments of the HHLO genomes directly from gastric biopsies, and its performance was compared with that of the assay targeting the *ureB* gene (Neiger *et al.* 1998). The aim was to develop an assay that could detect all known HHLO infections so the primers were designed by multiple sequence alignment of the 11 16S rDNA sequences of '*H. heilmannii*' types 1 and 2 as well as '*Candidatus* H. suis', *H. bizzozeronii*, *H. felis* and *H. salomonis* sequences currently held in GenBank. Application of the HHLO-16 assay to 12 different species of *Helicobacter* confirmed that only members of the HHLO group would be amplified. Other assays have been described for use on antral biopsies. For example, a '*Candidatus* H. suis'-specific PCR assay has been described that amplified product from porcine gastric biopsies (De Groote *et al.* 2000), while another assay for the *H. bizzozeronii*, *H. felis*, *H. salomonis* group was reported recently that successfully amplified specific product from canine gastric biopsies (De Groote *et al.* 2001). BLASTn analysis of each of the primer pairs demonstrated that the former assay would amplify only '*H. heilmannii*' type 1 sequences held in GenBank while the latter assay would amplify type 2 only. In contrast the HHLO-16 assay will amplify both types 1 and 2. As each type has been reported in the human stomach (Trebesius *et al.* 2001), this novel assay is more suitable for application to human gastric biopsies as it provides a rapid method of screening for all HHLOs in a single reaction.

Comparison of the HHLO-16 assay with the *ureB* assay by Neiger *et al* (1998) demonstrated that both assays generated product in 13/15 feline biopsies, but while three human biopsies were HHLO-16 PCR positive, only one human biopsy was positive by the *ureB* assay, and the histologically confirmed positive biopsy was PCR-

negative. This may indicate sequence variation in *ureB* between human and feline strains and suggests that the HHLO-16 assay is more sensitive for detection of HHLO infection in human samples. Moreover, a GenBank BLASTn search of the *ureB* primer sequences suggested that only 4/16 strains held in GenBank would be amplified by this assay. It seems likely that this *ureB* assay is '*H. heilmannii*' type 2-specific and thus lacks the broad specificity needed for initial testing of human infections. The HHLO-16 assay gave sharper single bands than the *ureB* assay that often generated non-specific bands.

### 4.3.3 The prevalence of HHLO infections in Southeast England

Application of HHLO-16 to 131 gastric biopsies (Groups 4 and 6) demonstrated a HHLO prevalence rate of 2.3 % in the series of patients in South East England. The incidence was higher than reported previously in other European countries (Ierardi *et al.* 2001;Mention *et al.* 1999) and the USA (Jhala *et al.* 1999;Mention *et al.* 1999). This observation suggests that histology-based studies have underestimated the true rate of infection or that the South East of England has a higher geographical prevalence. If the former were true, then patients who would benefit from specific eradication therapy are not being identified, highlighting the benefit of a PCR-based approach for detection as a more sensitive alternative to histology.

### 4.3.4 Evaluation of HpHh PCR assay for *H. pylori* and HHLO detection

The results presented show that the incidence of HHLO infection is significantly lower than that of *H. pylori* in the study population (2.3 % *vs.* 16.8 %) so it might not be cost effective to perform routine PCR tests for just HHLOs. The HHLO-16 assay was combined therefore in a multiplex format, initially with the PCR assay that amplified a fragment of the *H. pylori*-specific *vacA* gene, described in Chapter 3. The *vacA* primer combination was subsequently modified to allow co-amplification of

both *vacA* and the 16S rRNA genes. Evaluation of the HpHh assay on 230 human biopsies of known *H. pylori* and HHLO status demonstrated that the sensitivity and specificity of the HpHh assay was comparable to those of the individual PCR assays. In addition, DNA-seeding experiments demonstrated that both targets could be amplified simultaneously, so this system could potentially detect co-infections with both HHLOs and *H. pylori*. Analysis of the feline biopsies demonstrated that sensitivity of the HHLO-16 assay was slightly reduced, with one false negative result generated, in the multiplex format. As PCR reagent concentrations and cycling conditions were re-optimised to facilitate co-amplification of both targets, reduced efficiency of the HHLO-16 assay may have resulted for that sample from sequence variation at the primer binding sites. Comparison of sensitivity of detection of tenfold *H. felis* DNA dilutions (ranging from 100 ng to 1 fg) for both assay formats demonstrated that both uniplex and multiplex assays detected as little as 10 pg DNA, but the band intensity was lower for the product generated in the multiplex format. Thus the multiplex assay is only slightly less sensitive for HHLO detection than the uniplex format.

### 4.3.5 *H. pylori* and HHLO coinfection

Although human *H. pylori* and HHLO co-infection has been identified by histology (Ierardi *et al.* 2001), neither the incidence nor the possible significance of this in terms of disease development has been determined. Extensive optimisation of the novel multiplex assay presented here to allow co-amplification of targets from both species in artificially spiked samples could provide the tool to investigate this further. In this study, no evidence of co-infection was found. A total of 123 confirmed *H. pylori*-positive biopsies were tested by the HpHh assay, but generation of specific HHLOs was not observed in any of the biopsies that were PCR-positive for *H. pylori*. Thus it

was not possible to investigate the clinical significance of dual infection. Given the observed low incidence of HHLO infection in the population investigated, it could be concluded that the incidence of co-infections with *H. pylori* is comparatively rare in South East England. This study was limited by specimen availability but has nonetheless provided a powerful tool to allow future prospective investigations of larger study populations, to facilitate assessment of the true incidence of co-infection with both species and to promote understanding of the clinical significance of these.

### 4.3.6 Analysis of CLO test-positive biopsies

Biopsies from Group 7 had been identified as *H. pylori*-positive by rapid urease testing (CLO test). However, it should be noted that urease activity of '*H. heilmannii*' can also cause a CLO test colour change (McNulty *et al.* 1989). Consequently CLO test-positive results may be misinterpreted. CLO test-positive biopsies were analysed by the HpHh assay not only to investigate the prevalence of co-infections, as discussed above, but also to establish if any HHLO infections had been misdiagnosed as *H. pylori*-positive. As had been shown for other PCR detection assays (Chapter 3), the sensitivity of detection for the *vacA* assay was lower in these biopsies than those in Groups 4, 5, and 6. As discussed earlier (Chapter 3), DNA degradation may have occurred during transit. While no biopsies were identified that were CLO test-positive due to HHLO infection, the possibility of false negative results in 12 biopsies where specific DNA may have degraded cannot be fully excluded.

A recently reported novel fluorescent in-situ hybridisation (FISH) system demonstrated that '*H. heilmannii*' type 1 was the most prevalent in human infection, while type 2 and other novel types (3, 4 and 5) were less common, with mixed type infections also observed (Trebesius *et al.* 2001). Combination of the novel HpHh assay with the FISH technology would provide a comprehensive strategy for better

characterisation of HHLO infection in humans. The development of the HpHh assay provides a sensitive screening method to identify those biopsies positive for HHLOs that require additional, more complex testing by FISH for strain differentiation and characterisation. A recent report that identified *H. cinaedi* in the gastric biopsies of two patients (Pena *et al.* 2002) has raised the possibility that a broader range of helicobacters may be capable of colonising the human gastric mucosa than had been previously appreciated. As there is only one such report to date, further investigations aimed at detecting other species of *Helicobacter* including *H. cinaedi* will be necessary to establish the extent and significance of such infections.

## 4.4 Conclusions

The development and evaluation of a novel multiplex PCR assay for detection of HHLOs and *H. pylori* provides a sensitive means of direct detection of the principal pathogens causing gastric infection and disease in man. This is the first description of such an assay and its routine use would enable more accurate assessment of the prevalence of HHLOs infection in the dyspeptic population. Additionally this would facilitate studies to improve our understanding of the clinical significance and sources, such as domestic pets, of those infections in man, as well as enabling further investigation of HHLO/*H. pylori* co-infections. Future prospective analyses of gastric biopsies will now be possible to investigate larger study populations.

# Chapter 5: Real-time PCR determination of clarithromycin resistance directly from gastric biopsy samples.

## 5.1 Background

Chapters 3 and 4 have demonstrated the value of PCR for detection of helicobacters in gastric tissue. Another strength of PCR is that it allows further strain characterisation. As discussed earlier (section 1.10) clarithromycin (CLA) is a key component of *H. pylori* eradication triple therapy (de Boer and Tytgat 2000), but successful eradication is seriously compromised by the development of resistance to that antibiotic (Goodwin 1997;Megraud and Doermann 1998). CLA resistance rates of >10 % are reported in some Western European countries (Glupczynski *et al.* 2001). This highlights the need for rapid methods of antibiotic resistance monitoring, not only for individual patient management but also to facilitate enhanced surveillance for detection of temporal trends and regional variations in prevalence rates.

The mechanism of CLA activity and development of resistance is described in detail in section 1.11.2. As discussed in section 1.11.3, most molecular tests developed thus far for detection of the three most common mutations responsible for CLA resistance (A2142G, A2143G and A2142C) require multiple reactions and have been applied principally to *H. pylori* cultured from gastric biopsies (Stone *et al.* 1997;Taylor *et al.* 1997;Versalovic *et al.* 1996) The real-time PCR based probe hybridisation assay (LC-CLA) described in this chapter was initially shown to differentiate accurately between sensitive and resistant cultures of *H. pylori* (Gibson *et al.* 1999).

The aims of the study presented in this chapter were:

1. To develop the application of a real-time LightCycler PCR-based approach for determination of CLA resistance directly from gastric biopsies.
2. To investigate the utility of this assay as an alternative to culture-based CLA susceptibility testing methods.

## 5.2 Results

### 5.2.1 Optimisation of LC-CLA assay for application to gastric biopsies

The real-time LC-CLA assay described in section 2.20.5 developed for the Idaho LightCycler was applied initially to ten DNA extracts: five from *H. pylori* cultures and five from matched gastric biopsies, following the reported protocol (Gibson *et al.* 1999). Increased SYBR Green 1 fluorescence, indicating amplicon generation, was observed in DNA extracts from all five cultures but in only two of the five biopsies. The experiment was repeated using raised $MgCl_2$ concentrations (4.0 mM, 5.0 mM and 6.0 mM) but this did not improve the sensitivity. Next, the number of amplification cycles was increased from 50 to 75, which resulted in the generation of specific PCR product in all five gastric biopsy DNA extracts. These modified assay parameters were applied in all subsequent analyses.

### 5.2.2 Determination of CLA resistance from gastric biopsies by LC-CLA assay

The LC- CLA assay (section 2.20.5) was applied to a total of 259 gastric biopsies, 87 of which were *H. pylori*-positive by culture and/or histology (see Chapter 3). Biopsies were selected from previously defined Groups 2 (n = 39), 3 (n = 51), 4 (n = 121), 5 (n = 39) and 6 (n = 9). Examples of the melting peaks generated and sequences of control strains in relation to the probe sequence are provided in Figure

5.1. Results generated by this assay when applied to five distinct gastric biopsy groups are presented in Table 5.1.

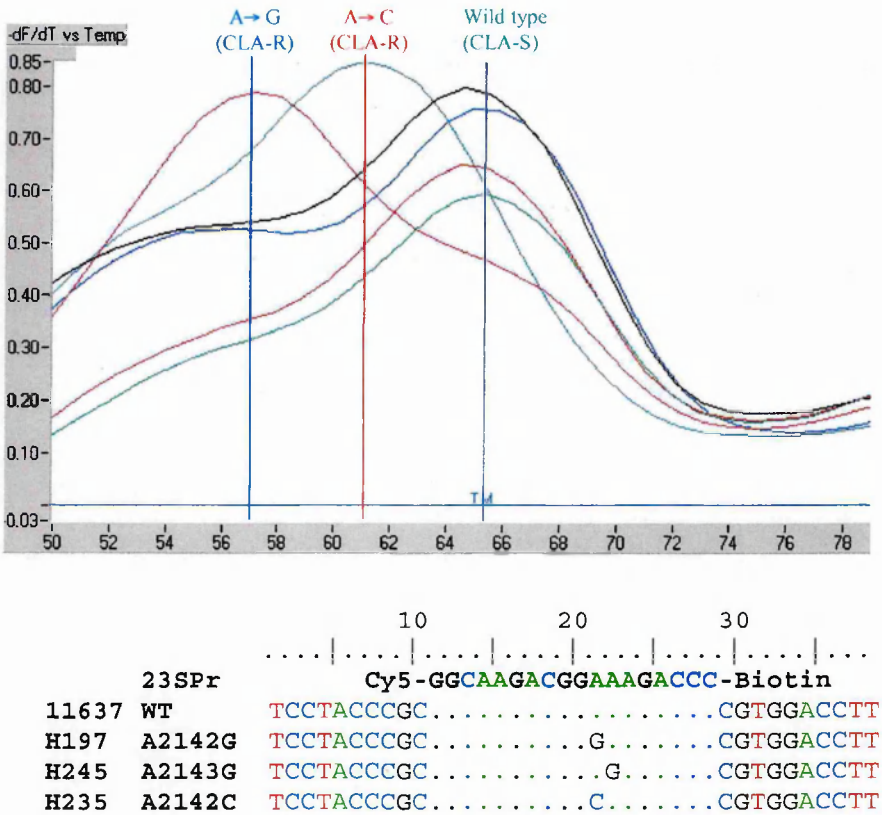



**Figure 5.1: Examples of LC-CLA assay probe melting peaks generated directly from gastric biopsies containing *H. pylori* 23S rDNA that is wild type, or has A2142G, A2143G or A2142C mutations, and sequence alignment of 23SPr with control strain sequences.**

**Table 5.1: Performance of LC-CLA assay to determine CLA susceptibility direct from gastric biopsies selected from five previously defined groups (Chapter 3).**

| 23S rDNA mutation | Gastric biopsy Group | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2 (n=39) | | 3 (n=51) | | 4 (n=121) | | 5 (n=39) | 6 (n=9) |
| | + | - | + | - | + | - | + | + |
| **Wild type** | 4 | 0 | 10 | 1* | 11 | 1* | 36 | 6 |
| **A→G** | 1 | 0 | 0 | 1† | 1 | 0 | 2 | 2 |
| **A→C** | 0 | 0 | 0 | 2† | 1 | 0 | 0 | 0 |
| **PCR negative** | 4‡ | 30 | 3‡ | 34 | 4‡ | 103 | 1‡ | 1‡ |

Biopsies were identified as *H. pylori* positive (+) or negative (-) by culture and/or histology.

*Biopsies were culture & histology negative but *H. pylori* PCR-positive (Chapter 3).

†Biopsies were positive for 16S rDNA *H. pylori*-specific PCR assay (Chapter 3).

‡All biopsies were falsely negative by at least one of three *H. pylori*-specific PCR assays (Chapter 3).

CLA susceptibilities were determined by 23S rDNA analysis in 85.1 % (74/87) of *H. pylori*-positive biopsies tested. DNA extracts from 67 biopsies produced melting peaks characteristic of a CLA sensitive genotype, as did two additional biopsies that were negative for culture and histology but were demonstrated to be *H. pylori* PCR-positive, as described in Chapter 3. Repeat PCR analyses were necessary for five samples to generate sufficient product to allow probe hybridisation melting point analysis, but amplification of 23S rDNA was not successful for 13 culture-positive biopsy specimens. DNA from 6/87 *H. pylori*-positive biopsies generated melting peaks indicative of a CLA resistant genotype with an A→G mutation and 1/87

generated a peak suggesting resistance due to an A→C mutation. Melting peaks were generated in five biopsies that were *H. pylori*-negative by culture and histology. Overall specificity of application of this assay to gastric biopsies was 97.1 %.

### 5.2.3 Determination of CLA susceptibility in matched culture by phenotypic and by genotypic (LC-CLA assay) methods

Matched isolates were available for 42 of the 84 gastric biopsies originally identified as *H. pylori*-positive by culture. For these 42 isolates, CLA susceptibilities were determined by E-test and by disk diffusion. For the remaining culture-positive biopsies, the results of disk diffusion tests performed by the primary isolating laboratory (Chelmsford PHL) were obtained. Altogether CLA susceptibilities of matched cultures were available for 79/84 patients. For five of the biopsies that generated a melting peak indicative of a 23S rRNA mutation, matched available cultures were also analysed by the LC-CLA assay, as were 15 other representative cultures grown from biopsies where melting peaks produced had suggested a wild type gene. A summary of the results generated in *H. pylori*-positive gastric biopsies by assay LC-CLA compared with the CLA susceptibilities determined for matched cultures by both genotypic and phenotypic methods is presented in Table 5.2.

Overall, antibiotic susceptibility testing by disk-diffusion or E-test of 62 available matched *H. pylori* cultures or laboratory reports confirmed that isolates from 60 biopsies that generated a melting peak curve indicative of a wild-type 23S rDNA sequence were CLA sensitive. The remaining two cultures comprised mixed populations of CLA sensitive and resistant phenotypes. E-test results on six available cultures matched with the seven biopsies that had generated peaks indicative of CLA resistance confirmed that five of these were CLA resistant (MIC $\geq$ 96 mg/ml).

Analysis of corresponding DNA extracts by the LC-CLA assay confirmed the identity of mutation types A→G (n = 4) and A→C (n = 1) originally detected in matched biopsies. The remaining isolate was CLA sensitive by E-test, in spite of an apparently resistant (A→G) LightCycler profile generated from gastric biopsy.

**Table 5.2: Details of CLA susceptibilities determined genotypically from 87 *H. pylori* positive gastric biopsies and from 20 matched cultures compared with available CLA resistotypes determined phenotypically**

| **Genotypic LC-CLA susceptibility test** | | | **Phenotypic CLA susceptibility test*** | | |
|---|---|---|---|---|---|
| **23S rRNA gene mutation** | **Gastric biopsy** | **Matched culture†** | **Culture data available** | **Sensitive** | **Resistant** |
| **Wild Type** | 67 | 15 | 62 | 60 | 2‡ |
| **A→G** | 6 | 4 | 5 | 1 | 4 |
| **A→C** | 1 | 1 | 1 | 0 | 1 |
| **PCR negative** | 13 | 0 | 11 | 10 | 1 |

*Disk diffusion and/or E-test.

†Number of 23S rDNA mutations determined from biopsies that were confirmed by matched culture LC-CLA assay analysis also

‡Mixed populations of CLA sensitive and resistant strains

## 5.3 Discussion

### 5.3.1 Determination of CLA resistance

CLA is a key component of most current triple therapy regimes for treatment of *H. pylori* infection, but resistance to this drug dramatically decreases the chance of

successful eradication (Dore *et al.* 2000a). The identification of specific point mutations, namely A2142G, A2143G or A2142C, in the 23S rRNA gene (Occhialini *et al.* 1997;Stone *et al.* 1997;Taylor *et al.* 1997;Versalovic *et al.* 1996) has enabled development of molecular tests that allow determination of CLA resistance direct from biopsies, without the requirement for culture (Bjorkholm *et al.* 1998). As discussed in section 1.11.3, at the time that this study was conducted, the majority of these tests were based on the principle that the mutated sequences create novel recognition sites for restriction enzymes (REs). Although PCR-RFLP provides information on the position of the mutation, it is time consuming, relatively labour intensive and until recently (Menard *et al.* 2002), the A2142C mutation could not be detected. Furthermore, some studies reported poor sensitivity (64 %) when applied to gastric biopsies (Sevin *et al.* 1998).

Additionally, other probe hybridisation methods, described in section 1.11.3.3, required multiple (three or more) reactions, adding to the test time and costs incurred (Maeda *et al.* 2000;Marais *et al.* 1999;Trebesius *et al.* 2000). The LC-CLA assay described in this chapter was the first to use the LightCycler to provide a test that was simple and more rapid than tests available, and enabled all three common mutations to be detected in a single reaction (Gibson *et al*, 1999). This chapter describes the first application of this real-time PCR based probe melting point hybridisation assay direct to gastric biopsies, for determination of CLA susceptibility without the requirement for culture. Reactions were performed in a single reaction tube in less than 1 h, so avoiding the risk of cross-contamination associated with molecular PCR-based assays and the delay of culture-based susceptibility testing.

### 5.3.2 Sensitivity of LC-CLA assay

Application of the modified assay to 87 *H. pylori*-positive gastric biopsies from Groups 2 – 6 (defined in Chapter 3), demonstrated that information on the CLA susceptibility of the infecting strain could be determined in 85.1 % of specimens. Thirteen culture-positive biopsies were negative for this test, with the highest proportion of falsely negative results observed in biopsy Groups 2 and 3 that had undergone prolonged incubation at room temperature prior to DNA extraction. As discussed in Chapter 3, most of these samples had been falsely negative by at least one PCR detection assay, suggesting that specific target DNA may have degraded. Certainly, the detection rate increased to 90.8 % if these two groups were excluded on the grounds that transport had been sub-optimal. In some cases, the sensitivity of the LC-CLA assay may have been adversely affected by sampling error; the target copy-number contained in the 1 µl DNA examined could be extremely low in some biopsies. Further evidence to support this hypothesis was provided by the initial observation that fifty PCR cycles were insufficient to generate enough product for further analysis, with no increase in SYBR Green 1 fluorescence being observed until 45-50 cycles for many biopsies. As described in section 2.20.2.1, measurement of the cycle number at which this fluorescence increases exponentially is the basis for quantification by the LightCycler, when compared with standard DNAs of known concentration. Given that PCR product was generated from the 10 ng DNA controls included in each run at approximately 35-40 cycles, this demonstrated that the biopsies contained significantly less specific target. Repeat testing of five gastric biopsies that had initially failed to amplify specific product was subsequently successful, demonstrating the potential problems of sampling errors between runs when target levels are so low. The simple digestion method of DNA extraction for

these biopsies, discussed in Chapter 3, was selected as it was sufficiently rapid to enable same-day diagnosis of *H. pylori* infection and determination of CLA susceptibility. However, it did not contain a precipitation/resuspension step to concentrate extracted DNA. Sensitivity could be improved by adopting a more complex method of DNA extraction such as phenol chloroform extraction or use of a commercial kit. Alternatively the assay could be modified to a nested PCR format, although this would increase the total processing time and raise the risk of sample contamination. Assay sensitivity could be reduced also by low levels of PCR-inhibitory substances that may affect the efficiency of a LightCycler assay to a greater extent than that of the conventional assays.

### 5.3.3 Specificity of the LC-CLA assay

Of the 172 culture and histology-negative biopsies analysed, melting peaks were generated in only five specimens (2.9 %). The two biopsies that generated peaks consistent with a CLA sensitive phenotype had been demonstrated to be positive by *H. pylori*-specific PCR assays applied in the study described in Chapter 3. This corroborative evidence suggests that these are genuine positives and that tests on these biopsies were falsely negative by culture and histology, neither of which are 100 % sensitive (El Zimaity 2000;Perez-Perez 2000). This demonstrates that PCR is not only able to detect *H. pylori* infections missed by other diagnostic methods, but provides information on CLA susceptibility that could not be obtained by any other conventional method. The remaining three biopsies produced melting peaks that were initially interpreted as corresponding to A→C or A→G mutations. However, results presented in Chapter 3 indicated that two of these biopsies had been PCR-positive only by a single *H. pylori* specific assay targeting 16S rRNA. These discrepant results were thought to be due to overgrowth of other bacterial species while the

biopsies were in transit. The LC-CLA assay amplifies 23S rDNA, homologues of which are universally present in all bacteria (Stackebrandt and Goodfellow 1991). BLASTn analyses suggested that the assay primers were *H. pylori*-specific, but other contaminating bacterial DNA that has not yet been characterised by sequencing, and so is not available in GenBank, may have been amplified in these cases. The sequence of these amplicons would differ from that of *H. pylori*-specific product and mismatch between probe and template would lower the probe dissociation temperature, as was observed. Although such melting peaks generated could be misinterpreted as infection with a resistant strain, it is important to note that false results were only generated in biopsies that had been subjected to considerable delays in transport and that no falsely positive results were generated for any other biopsy group. Furthermore, interpretation of these results in conjunction with those of the *H. pylori*-specific PCR were sufficient to highlight that these results were discrepant, therefore the likelihood of mis-diagnosis in a routine clinical diagnostic setting is low.

### 5.3.4 Accuracy of CLA resistotype determined by LC-CLA assay

Overall, the LC-CLA assay accurately determined CLA resistotype in most (95.6 %) *H. pylori*-positive biopsies. For two of the three cases where biopsies generated profiles that did not correspond to matched isolate CLA susceptibility tests, this was due to mixed CLA resistotype infection. Although rare, CLA resistant strains that lack mutations A2142G, A2142C and A2143G are documented (Hulten *et al.* 1997;Gibson *et al.* 1999;Fontana *et al.* 2002). These would not be detectable by any of the molecular methods described for CLA susceptibility testing (section 1.11.3), including the LC-CLA assay. However analysis of the two CLA resistant sub-populations by this test demonstrated that both contained A→G mutations, thus discrepant results between matched cultures and biopsies were not due to absence of

the common mutations associated with CLA resistance. In most mixed infections in this study, the proportion of sensitive population exceeded that of the resistant population, by an estimated factor of > 100 in these two examples. The amplification of 23S rDNA of the sensitive sub-population for which more starting template was available would be more efficient during PCR as these would enter the exponential phase of the reaction earlier than the resistant population. At the end of the PCR stage, there would be significantly higher levels of amplicon from the sensitive population and, as the probe was exactly complementary to the wild-type template, preferential hybridisation of probe to this more-abundant template would occur, leading to generation of a peak indicative of CLA sensitivity. These results may highlight a limitation of single probe-based approach, namely that sensitivity may be insufficient to detect a very low level CLA resistant sub-population. Furthermore, a LightCycler profile indicative of CLA resistance was consistently generated from one gastric biopsy, where the matched culture tests identified a CLA sensitive population. A possible explanation for this is that the *in vivo* infection was a mixture of both CLA resistant and sensitive forms but only the latter was isolated on culture. Similar discrepancies between mutation detection and phenotypic susceptibility testing have been reported previously (Matsuoka *et al.* 1999). This suggests that even culture based CLA susceptibility testing may not always detect mixed strain infections. It is recognised that development of antibiotic resistance can lead to decreased fitness of a bacterial population (Andersson and Levin 1999). The possibility that efficiency of *in vitro* culture of some resistant *H. pylori* strains may be slightly impeded cannot be excluded, particularly if competing with a more robust sensitive co-existing population. A PCR-based approach has the advantage that any such differences in strain fitness would not affect the sensitivity of detection or probe-based analyses.

Mixed susceptibility infections were not investigated in the original assay evaluation (Gibson *et al.* 1999), but the problem could be overcome possibly by the development of additional probes complementary to each mutation type as these would bind preferentially to any exactly matched sequence, even if present at comparatively low levels. The Idaho model of the LightCycler used in this study had the limitation that only two channels were available for fluorescence measurement, and these were set at wavelengths which were optimal for dyes SYBR Green 1 (channel F1) and Cy5 (channel F2). This restriction prevented the development of multiplex assays with specific probes labelled with different fluorescent dyes. Thus for CLA susceptibility testing, four separate reactions with each different probe would be necessary to exclude all possibility of a mixed infection. Such an approach would greatly increase the cost, workload and time for each test to an extent that would be difficult to justify, considering the high level of accuracy demonstrated for the single probe-based approach.

At the same time that this study was conducted Matsumura and colleagues reported a similar approach for the LightCycler (Roche Instrument) (Matsumura *et al.* 2001). This assay successfully determined CLA susceptibility from all biopsies tested and was also highly accurate. As discussed earlier (section 2.20), the fluorescent chemistry of the Roche LightCycler differs from that of the Idaho model, requiring two probes per reaction to achieve FRET for monitoring of probe hybridisation melting point. For the LC-CLA assay, a single probe was sufficient to distinguish 23S rDNA that was wild type or contained either A→G or A→C mutations. In contrast the assay described by Matsumura and colleagues is more complex and also more expensive, as three separate reactions containing one of three probes that were complementary to the wild type, the A2142G or the A2143G 23S rRNA genotype

were required. However, although this assay was unable to detect the A→C mutation, it does have the advantage of providing information on the position of the A→G mutation, that may be related to MIC (Alarcon *et al.* 2000;Dzierzanowska-Fangrat *et al.* 2001;Piana *et al.* 2002;Pina *et al.* 1998;Versalovic *et al.* 1997). A small temperature difference between melting peak and position of the A→G mutation was observed in the LC-CLA assay (J. R. Gibson, personal communication), but this was not marked enough to allow reliable definition of mutation type.

Since this study was conducted, the LightCycler Idaho model has been largely superseded by the Roche model and novel assays that identify all three common mutations that confer CLA resistance have been developed for the latter model (Lawson *et al.* 2002). Early studies such as the one described in this chapter and elsewhere (Matsuoka *et al.* 1999) were nonetheless invaluable in demonstrating the potential of the LightCycler technology in this field and have stimulated the development of novel assays that are currently undergoing evaluation in Germany and France (U. Reischl, personal communication). Recently one such assay was reported that can detect mixed CLA susceptibility infections directly from biopsies in a single reaction (Oleastro *et al.* 2003). Thus application of a LightCycler-based approach for antibiotic susceptibility testing could become more widespread in the future. Tetracycline (TET) resistance was shown recently to occur in *H. pylori* by point mutation in the 16S rRNA gene (Trieber and Taylor 2002). This simple mechanism of antibiotic resistance would be suitable for testing by an assay analogous to LC-CLA. The development of this in the future would provide an additional tool for enhanced surveillance of antibiotic resistance in *H. pylori*.

## 5.4 Conclusions

CLA susceptibility can be determined accurately in real-time direct from gastric biopsies, so avoiding the associated delays of culture. This study was among the first to apply a LightCycler assay to gastric biopsies for this purpose and demonstrates the power and versatility of PCR as a tool for management of *H. pylori* infection. Rapid provision of CLA susceptibility information could have a significant impact on patient management, in terms of turnaround times, appropriate antibiotic prescription and ultimately treatment outcome.

# Chapter 6: Determination of *vacA* genotype direct from gastric biopsies in a novel multiplex PCR assay format.

## 6.1 Background

PCR has proved to be a key technique for the investigation of potential virulence factors in a range of bacterial infections (Lehoux *et al.* 2001;van Doorn 2001;Weinstock 2000). In the case of *H. pylori*, the observed variations in disease progression between patients are thought to be at least partially attributable to differences in bacterial virulence (van Doorn 2001). The vacuolating cytotoxin gene (*vacA*) is one such marker of enhanced virulence potential. As described earlier (section 1.12.1), the mosaic structure of *vacA* comprises two families of allelic variants of the signal sequence region (s1, s2) and of the mid-region (m1, m2) (Atherton *et al* 1995). Although some studies have reported an association between *vacA* genotype and disease outcome (van Doorn *et al* 1998, Kidd *et al* 1999, Rudi *et al* 1999), others have not confirmed this (Go *et al* 1998, Gunn *et al* 1998, Gold *et al* 2001). Further study of larger populations from different geographical areas will be essential to improve understanding of the significance of *vacA* genotype in terms of virulence potential. As discussed (section 1.12.1.2), most reports to date have defined *vacA* genotype by performing two or more PCR reactions; such studies have adopted PCR assays based on specific primers designed by Atherton *et al* (1995, 1999) (Ito *et al* 1997, Evans *et al* 1998, Gunn *et al* 1998, Strobel *et al* 1998, Kidd *et al* 1999).

The aims of the study presented in this chapter were:

1. To develop PCR assays to facilitate high-throughput examination of *H. pylori* *vacA* allelic type direct from gastric biopsies.

2. To apply developed assays to examine, and improve understanding of, the relationship between *vacA* genotype and disease status in South East England.

## 6.2 Results

### 6.2.1 Development and optimisation of multiplex *vacA* genotyping PCR assay

Primers that had been developed previously (Atherton *et al* 1999) to allow determination of *vacA* signal and mid region allelic types in two separate conventional PCR reactions, as described in section 2.18.1.1, were initially combined in a single reaction in a 1:1 ratio. Application of this to 15 cultures of known *vacA* genotype successfully generated the expected type in 13/15 samples, but in some cases the mid region amplicon was either not generated, or was of considerably lower intensity than that of the signal region product. Alteration of the signal:mid region primer ratio to 1:1.6 along with a raised $MgCl_2$ concentration (2.0 mM) promoted generation of the mid region as well as the signal region products in all 15 cultures. This optimised multiplex format, described in section 2.18.1.2 was evaluated further as described in subsequent sections.

### 6.2.2 Performance of multiplex *vacA* genotyping assay on DNA from culture

Accuracy of the multiplex *vacA* genotyping PCR assay was evaluated on DNA extracted from a total of 22 cultures of *H. pylori* for which the *vacA* genotype had been determined by the original two-step system described in section 2.18.1.1. The multiplex PCR assay developed successfully generated products of sizes relating to signal region types s1 (259 bp) and s2 (286 bp), and mid region types m1 (567 bp) and m2 (642 bp) (Figure 6.1). The expected s/m profile was generated for 21/22 culture DNA extracts of known *vacA* genotype (ten s1m1, seven s1m2 and four s2m2). In one strain (H2966) known to be type s1m1, the expected 259 bp band

corresponding to type s1 and a larger fragment of approximately 1.6 kb were generated but no band corresponding to either m type was observed.

### 6.2.3 Determination of *vacA* genotype direct from gastric biopsies

A total of 123 gastric biopsies, positive for *H. pylori* by either culture and/or histology or by CLO test were selected from the biopsy Groups 4 (n = 13), 5 (n = 39), 6 (n = 18) and 7 (n = 53), defined in Chapter 3. All were tested by both uniplex and multiplex PCR assays for determination of *vacA* genotype. Descriptions of disease status and macroscopic appearance of the upper gastrointestinal tract recorded for every patient at the time of endoscopy were available for 102/123 patients.

Initial application of the multiplex system to 15 gastric biopsies and 15 matched cultures generated exactly correlating genotypes that were easy to interpret in all cases. Examples of these are illustrated in Figure 6.1. Of the 123 *H. pylori*-positive gastric biopsies tested, *vacA* genotype was determined in 101 (82.1 %) using the two-step system (Atherton *et al* 1999), and in 100 (81.3 %) biopsies by the novel single-step approach.

The *vacA* genotypes were generated in 67/70 (95.7 %) gastric biopsies where *H. pylori* status had been determined by culture and/or histology (Groups 4 – 6) and in 33/53 (62.3 %) of biopsy Group 7 that were *H. pylori* positive by CLO test alone. Sensitivity in biopsy Group 7 was only slightly higher (64.2 %) when signal and mid-region testing were performed by the original two-step Atherton method.

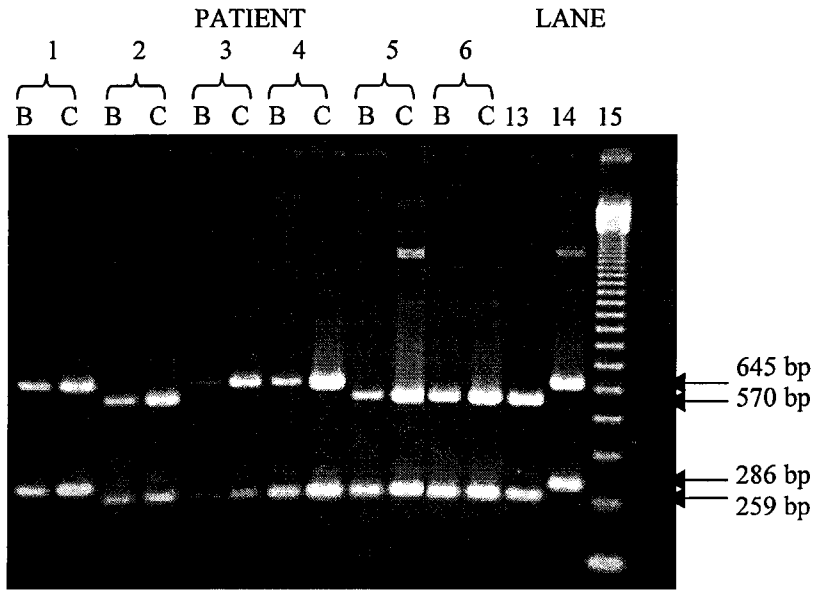



**Figure 6.1:** ***vacA* genotypes determined by multiplex PCR assay direct from human gastric biopsies (B) (Groups 5 and 6) compared with matched cultures (C).** Patient 1, type s2m2, Patients 2, 5 & 6, type s1m1, Patients 3 & 4 type s1m2. Lane 13, s1m1 control (NCTC 11637). Lane 14, s2m2 control (Tx30a). Lane 15, 123 bp molecular weight marker.

### 6.2.4 Distribution of *vacA* genotypes according to disease

Of the 100 *vacA* genotypes determined from gastric biopsies, profile s1m1 accounted for 43.0 % of samples tested while s1m2 and s2m2 accounted for 31.0 % and 26.0 %, respectively (Table 6.1). Accompanying clinical information was available for 35, 27 and 23 patients infected with *H. pylori* of genotypes s1m1, s1m2 and s2m2, respectively. Of the 35 s1m1 genotypes identified, 9 (25.7 %) were associated with peptic ulcer disease (PUD) and 74.3 % were from patients in the non-ulcer dyspepsia (NUD) group. In contrast, only 8.1 % of type s1m2 and 8.7 % of type s2m2

genotypes were identified in patients with PUD. The higher proportion of type s1m1 in PUD-associated strains compared with NUD strains is illustrated in Figure 6.2.

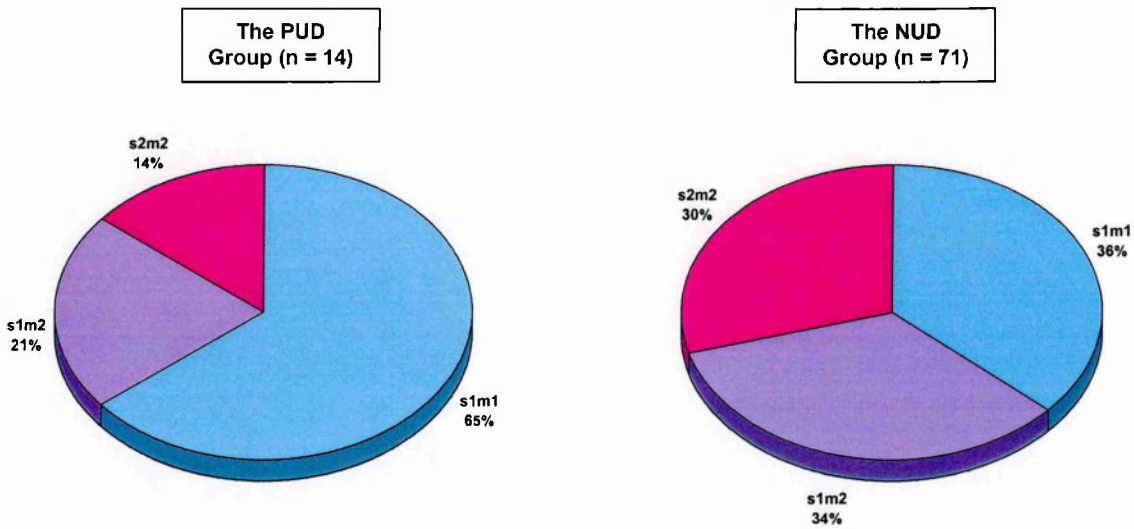



**Figure 6.2 Schematic representation of the proportions of *vacA* genotypes in the peptic ulcer disease (PUD) groups and non-ulcer dyspepsia (NUD) groups.**

Additionally, further examination of the NUD group demonstrated that type s1m1 was found in biopsies from 15/29 patients with macroscopic signs of gastritis and/or duodenitis recorded at the time of endoscopy, that could potentially lead to ulcer development. In contrast, only 5/26 (19.2 %) of isolates of type s2m2 were associated with macroscopic gastroduodenal inflammation (Table 6.1). Statistical analysis by Fisher's exact test (section 2.25.3) of results for patients where the disease status was known demonstrated that the proportion of patients with gastroduodenal symptoms (inflammation or ulcer) infected with strains of type s1m1 was significant (p value 0.008), relative to patients who were either infected with type s1m2 or s2m2, or for

whom no disease or other conditions (oesophagitis, hiatus hernia) were documented at the time of endoscopy. The distribution of *vacA* genotype in relation to specific disease status is presented in Table 6.1.

**Table 6.1: Distribution of *H. pylori vacA* genotypes determined direct from 100 gastric biopsies in relation to patient disease status**

| | Disease Status | *vacA* Genotype | | | Total |
|---|---|---|---|---|---|
| | | s1/m1 | s1/m2 | s2/m2 | |
| PUD Group | Gastric ulcers | 3 | 1 | 2 | 6 |
| | Duodenal ulcers | 5 | 2 | 0 | 7 |
| | Gastric and duodenal ulcers | 1 | 0 | 0 | 1 |
| NUD Group | Gastritis/duodenitis only | 15 | 9 | 5 | 29 |
| | Normal* endoscopy | 7 | 7 | 9 | 23 |
| | Oesophagitis | 3 | 6 | 6 | 15 |
| | Hiatus hernia only | 1 | 2 | 1 | 4 |
| | Information not available | 8 | 4 | 3 | 15 |
| | Total | 43 | 31 | 26 | 100 |

*All patients had dyspeptic symptoms, but no macroscopic signs of disease were recorded at time of endoscopy

## 6.3 Discussion

The use of *vacA* genotyping methodologies is a key feature in studies investigating virulence potential of *H. pylori* from geographically diverse localities (Ito *et al* 1997, Evans *et al* 1998, Go *et al* 1998, Gunn *et al* 1998, Strobel *et al* 1998, van Doorn *et al*

1998, Kidd *et al* 1999, Rudi *et al* 1999, Gold *et al* 2001). Many different PCR-based and line probe-based assays have been described for genotyping, some requiring multiple reactions (Atherton *et al* 1995, Ito *et al* 1997, Rudi *et al* 1998, Strobel *et al* 1998, van Doorn *et al* 1998) while others can be performed in just two separate PCR tests for the signal and for the mid-region (Atherton *et al* 1999). One other single-step *vacA* genotyping assay has been described previously that used the Atherton primers to amplify the signal region but novel primers to target the mid-region (Han *et al* 1999). In contrast, the work in this chapter describes a novel one-step system that enables rapid and convenient genotyping of both regions of *vacA*; the approach was to combine and re-optimise two published PCR assays. The sensitivities and specificities of the latter assays had been evaluated extensively as two separate reactions on both cultures and biopsies by the HRU and by other groups (Atherton *et al.* 1999;Kidd *et al.* 1999;Owen *et al.* 2002).

### 6.3.1 Performance of *vacA* genotyping assay (single-step *vs* two-step format)

Han *et al* (1999) reported that their multiplex assay failed to determine *vacA* type in 7 % of isolates tested. Strains were defined as untypeable if no product was generated or if both m types (m1 and m2) or s types (s1 and s2) were observed. Previously, routine determination of *vacA* genotype in >400 strains in the HRU by the two-step PCR test (Atherton *et al* 1999) demonstrated that a much smaller proportion of strains could be classed as untypeable by these criteria (approximately 2.3 %, R. J. Owen, personal communication). Most "untypeable" isolates were dual m type patterns of both m1 and m2. Investigation of multiple m types by genotyping single colony picks showed that dual m types resulted from mixed strain infections rather than an inability of the assay to correctly determine *vacA* type (R. J. Owen, personal communication). Mixed type infections have been demonstrated in line probe assay-based studies also

(van Doorn *et al.* 1998;van Doorn *et al.* 1999). Thus if dual types are excluded from this definition of untypeable, the two *vacA* PCR assays that were combined in a single assay achieves high-level typeability. Furthermore, application of the novel multiplex system to DNA extracts from isolates that had been genotyped by the original two-step system correctly identified *vacA* genotype in 21/22 isolates, demonstrating that this new format determined *vacA* profiles with high-level accuracy.

For isolate H2966, a larger fragment (>1.6kb) was generated but no band corresponding to m type observed. Determination of m type in a separate PCR reaction for this isolate generated a very weak intensity band of 567 bp (m1) suggesting that the efficiency of this reaction was sub-optimal, possibly due to sequence variation at the primer binding site. Efficiency of this PCR would be further reduced in a multiplex format due to, for example, competition for reagents with the s typing reaction, and this may account for the unsuccessful amplification of m type in this isolate. However, this is likely to be a rare occurrence, as it was not observed in any of the other cultures or biopsies tested in this study.

### 6.3.2 Determination of *vacA* genotype from gastric biopsies

There are several reports of accurate and sensitive *vacA* genotyping direct from gastric biopsies (Rudi *et al*, 1998, Mattar and Laudanna 2000). This is a significant development as it not only overcomes the considerable delays of culture, allowing rapid determination of *vacA* genotype, but it can also provide additional strain information from biopsies where the *H. pylori* is no longer viable. The performance of the novel multiplex PCR system developed in this study was assessed by examination of four different gastric biopsies groups, defined in Chapter 3, that were of known *H. pylori*-positive status, determined in biopsy Groups 4 – 6 by culture and histology, and in biopsy Group 7 by CLO test alone. Application of the single-step

genotyping system to 15 biopsies from Groups 5 and 6 and to their matched cultures generated identical *vacA* types in each biopsy/culture pair. In addition, non-specific bands due to human DNA in gastric biopsies were rarely observed. Application of this assay directly to gastric biopsy therefore generates a profile that is both accurate and easy to interpret. Further testing of 55 additional biopsies from Groups 4 – 6 demonstrated that *vacA* genotype could be determined with high sensitivity (95.7 %).

Lower sensitivity (62.3 %) was observed for the CLO-test positive biopsies (Group 7) – a figure that was also lower than sensitivities reported in other studies where *vacA* genotype was determined direct from CLO-test positive gastric biopsies (Rudi *et al* 1998, Mattar and Laudanna 2000). Determination of *vacA* genotype in these biopsies by the original two-step approach was only marginally more sensitive (64.2 %), thus the low sensitivity observed was not due to poor performance of the multiplex system. As discussed in Chapter 3 the low sensitivities of PCR detection assays demonstrated in this biopsy group were proposed to be attributable to degradation of target DNA. However, while none of these biopsies contained viable organisms, it was still possible to determine the *vacA* genotype in a considerable proportion (62.3%) of this group.

It is evident that PCR-based systems provide an invaluable means of gaining additional strain information that would not be otherwise available from specimens that are not routinely cultured. This is particularly important for *H. pylori* as relatively few centres routinely diagnose infection by culture in the UK or in other European countries (Heep *et al* 2001). In contrast, CLO tests are used widely as they allow rapid detection of *H. pylori*, even though they provide no further strain information. This multiplex system therefore provides a rapid, accurate and economical mode of *vacA* genotype surveillance from a larger cross-section of the

dyspeptic community than would be available if relying on culture alone. This should facilitate more extensive and comprehensive studies of the relationship between *vacA* genotype and disease status and ultimately improve understanding of pathogenicity of *H. pylori* infection.

### 6.3.3 *vacA* genotype in relation to disease progression

Using gastric biopsies directly as a means of determining *vacA* genotype information demonstrated that s1m1 was the most prevalent genotype in South East England, while type s1m2 and s2m2 were less common. Although no exact correlation between genotype and disease status was observed, genotype s1m1 appeared to be more often associated with peptic ulcer disease and severe gastritis or duodenitis while types s1m2 and s2m2 were more frequently observed in patients who had other conditions or were macroscopically normal at endoscopy. As the survey was relatively small, no firm conclusions can be made regarding the role of *vacA* genotype in relation to disease progression, although statistical analyses suggested that this association was significant. These observations support reports from other workers that type s1/m1 strains appear to be more virulent than s2/m2 strains (Gunn *et al* 1998, van Doorn *et al* 1998, Kidd *et al* 1999, Rudi *et al* 1999). The multiplex assay described and its potential to be applied directly to clinical samples will however provide the means to study larger populations to allow further evaluation of this relationship.

## 6.4 Conclusions

The single-step multiplex PCR reaction developed in this study has provided an alternative means of genotyping the signal and mid-regions of *vacA*, based on well-validated primers. The assay is rapid, highly accurate and also more convenient and

economical than existing assays, both in terms of labour and reagents. Additionally, this system can be applied directly to gastric biopsies analysed by the rapid urease (CLO) test, for which culture is not performed. This will facilitate further surveillance of *vacA* genotype and improve understanding of the role of this gene as a potential virulence factor in *H. pylori* infection.

# Chapter 7: Development and application of real-time PCR in the investigation of *H. pylori cagA* tyrosine phosphorylation motifs in relation to strain virulence

## 7.1 Background

The previous chapter demonstrated the value of PCR for rapid *vacA* genotype determination from bacterial isolates and clinical samples. The results presented supported the previous observation that no single pathogenicity factor has yet been proved to be uniquely associated with the ability of *H. pylori* to cause gastroduodenal ulcer disease or cancer (Blaser 1999).

As discussed in section 1.12.2, *cagA* is one of 31 genes encoded on a 40-kb pathogenicity island (PAI) present in a subset of strains (type I) that reportedly causes higher levels of inflammation than strains lacking *cag*PAI (Censini *et al.* 1996). Recent *in vitro* studies suggest that *H. pylori* exports CagA protein via a type IV secretion system encoded by the *cag* PAI (Backert *et al.* 2000;Odenbreit *et al.* 2000;Stein *et al.* 2000). CagA is then translocated into the gastric epithelial cells where it induces host cell kinases that phosphorylate tyrosine residues in CagA adjacent to the site of bacterial adhesion on the host gastric epithelial cells. This in turn activates eukaryotic signal transduction pathways, leading to actin reorganisation and pedestal formation (Stein *et al.* 2000). Three putative nucleotide tyrosine phosphorylation motifs (TPMs), designated A, B and C, in the CagA protein were predicted with the MOTIF algorithm (Odenbreit *et al.* 2000). The significance of these in terms of bacterial virulence has not been established to date.

The aims of the study presented in this chapter were:

1. To develop and evaluate novel real-time LightCycler PCR hybridisation assays for the detection of nucleotide sequence motifs in *cagA* corresponding to the amino acid TPMs A, B and C proposed by Odenbreit *et al* (2000).
2. To apply these assays to a set of 84 clinical isolates of *H. pylori*, all from one locality in England (mid-Essex), firstly to determine the prevalence of motifs A, B and C, and secondly to assess the significance of these in relation to disease severity.
3. To establish if the assays developed could determine *cagA* TPMs A, B and C directly from gastric biopsies.

## 7.2 Results

### 7.2.1 Bacterial strains

Eighty-four isolates of *H. pylori* included in the study originated from the HRU collection, cultured from antral gastric biopsies from dyspeptic patients undergoing routine upper gastrointestinal tract endoscopy at Broomfield Hospital (Chelmsford). No specific clinical selection criteria were applied for inclusion of patients in the study, but all isolates were reported previously to carry an intact *cag*-PAI (Owen *et al.* 2001) and *vacA* signal (s) and mid-region (m) type had been determined prior to this investigation. Isolates from five clinical disease groups, defined on the basis of endoscopic investigation, were examined: the duodenal ulcer (DU) group (n = 23 strains), the gastric ulcer (GU) group (n = 14 strains), the DU and GU group (n = 4 strains), the gastric neoplasia (GN) group (n = 2), and the non-ulcer dyspepsia (NUD) group (n = 41 strains). Similarly to the NUD group described in the previous chapter, the latter group was diverse as it contained patients diagnosed with chronic gastritis

and/or duodenitis, and/or oesophagitis, as well as endoscopically normal oesophagus, stomach and duodenum.

All 84 isolates were tested for TPMs A, B and C by three novel PCR assays (CagMotA, CagMotB and CagMotC), developed for the LightCycler, as described in section 2.20.6. As this was a collaborative study within the HRU, isolates were also tested for TPM-A and TPM-C by three novel PCR-RFLP assays that were developed and evaluated in a separate study, allowing comparison of the relative performances of real-time versus block PCR (data made available by kind permission of S. I. Sharp and R. J. Owen). Further evaluation of assays was performed by direct DNA sequencing of representative isolates, following the protocols described in sections 2.18.2.2 and 2.22.

### 7.2.2 Development and evaluation of *cagA* TPM-A LightCycler assay (CagMotA).

Probe *CagMotA* was designed to complement sequences of control strains NCTC 11638 and NCTC 12455 (strain 26695) that contain nucleotide sequences corresponding to the TPM-A described (Figure 7.1). Initial evaluation of this assay by testing control strain DNAs (Appendix A.2.3) demonstrated that strains containing the motif generated melting peaks indicating the highest probe-template dissociation temperature (approximately 65.0 °C) (Figure 7.1). Observed probe-template Tms of TPM-A positive strains varied between runs, ranging from 60.4 to 66.0 °C. In contrast, lower probe Tms were determined for reference strains lacking TPM-A, ranging from 55.6 to 61.5 °C (Figure 7.1). TPM-A positive strains were therefore always defined relative to the reference DNA samples.

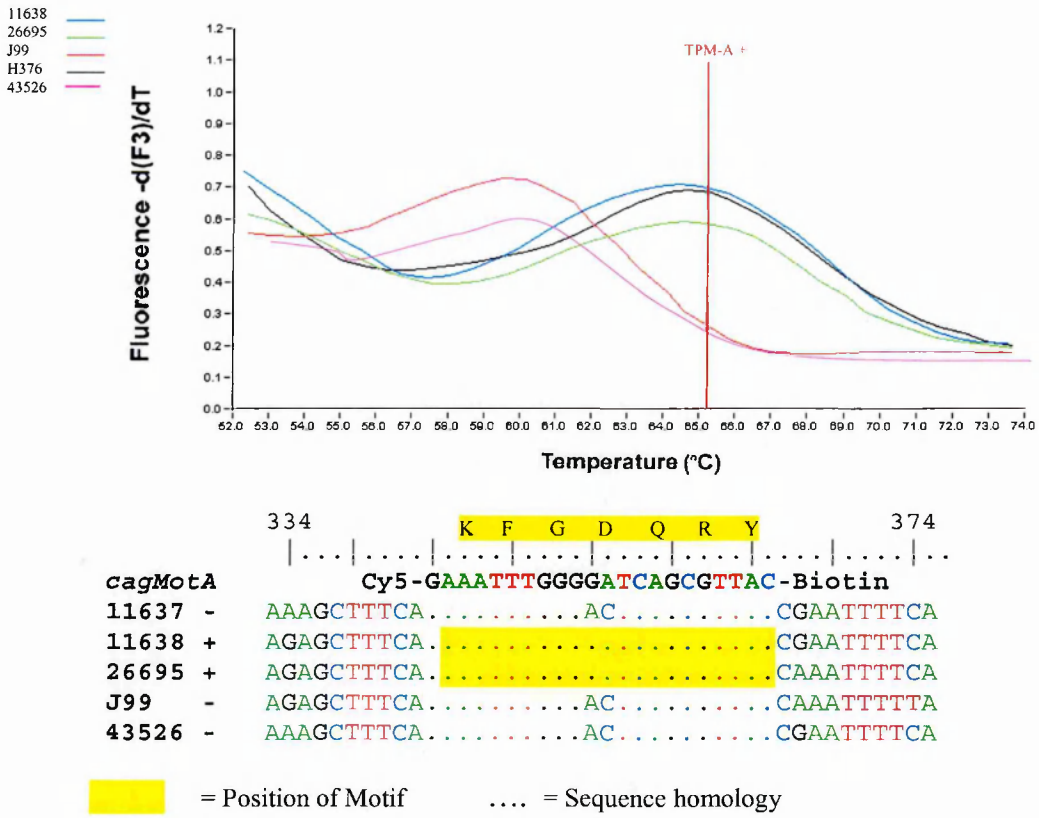



**Figure 7.1: Examples of melting curves generated by four *H. pylori* reference strains, and alignment of *CagMotA* with reference strain sequences.**

Performance of LightCycler assay CagMotA was evaluated by comparison of results obtained with those generated by two PCR-RFLP assays that differentiated TPM-A positive and negative strains on the basis of specific profiles generated following restriction of a 356-bp *cagA* fragment with either *Hinf*I or *Dde*I (S.I. Sharp, personal communication). Of the 84 isolates tested, 54 were TPM-A positive by all three assays. Two of these isolates were confirmed as TPM-A positive by sequencing also. Four isolates were negative by assay CagMotA but positive by both PCR-RFLP assays. Product amplification failed in the CagMotA assay in one other isolate but gave a positive result in both PCR-RFLP assays, while another isolate did not yield a

PCR product for RFLP analysis but was identified as TPM-A positive by assay CagMotA. The 24 isolates remaining were TPM-A negative by both approaches. However partial digestion of amplicon was observed in 19/24 cases for one of the PCR-RFLP assays, and Tms generated for some isolates by the CagMotA assay were only slightly lower than those observed for the control TPM-A-positive strains. Thus, the absence of TPM-A was also confirmed by sequencing in all 24 cases (data not shown). Overall, TPM-A was detected by at least one of the assays in 60 isolates (71 %), either singly (64 %) or in combination with other TPMs (7 %).

### 7.2.3 Development and evaluation of *cagA* TPM-B LightCycler assay (CagMotB).

Probe *cagMotB* was designed to complement the sequence of the reference strain 26695 that contained TPM-B (Appendix A.2.3, Figure 7.2). While most reference strains lacked TPM-B due to a 6-bp deletion at nucleotide 2698, the apparent absence of TPM-B in reference strain J99 is due to a single substitution only (A2693G) (Figure 7.2). TPM-B is positioned in the variable region of *cagA* (Odenbreit *et al.* 2000;Yamaoka *et al.* 1998). To ensure that any probe-template mismatch was due to absence of TPM-B and not due to inherent sequence variation, *cagMotB* was designed to span a region that commenced 12 bp upstream of the region associated with TPM-B (Figure 7.2). Preliminary testing of reference strains demonstrated that the Tm of probe *cagMotB* for TPM-B-positive strain 26695 was highest (approximately 67.0 $^{o}$C), while the observed Tms for reference strains lacking TPM-B were lowered according to the level of sequence variation (Figure 7.2). Strain J99 was most similar to the probe and so generated a peak indicating a slightly lower Tm than observed for 26695, while strains NCTC 11637 and ATCC 43526 that had the highest degree of mismatch generated low-intensity peaks of low Tm (Figure 7.2). A PCR-RFLP assay

could not be developed to test for TPM-B as the level of sequence variation in this region of *cagA* is high and a suitable recognition site for potential restriction enzyme (RE) digestion could not be found.

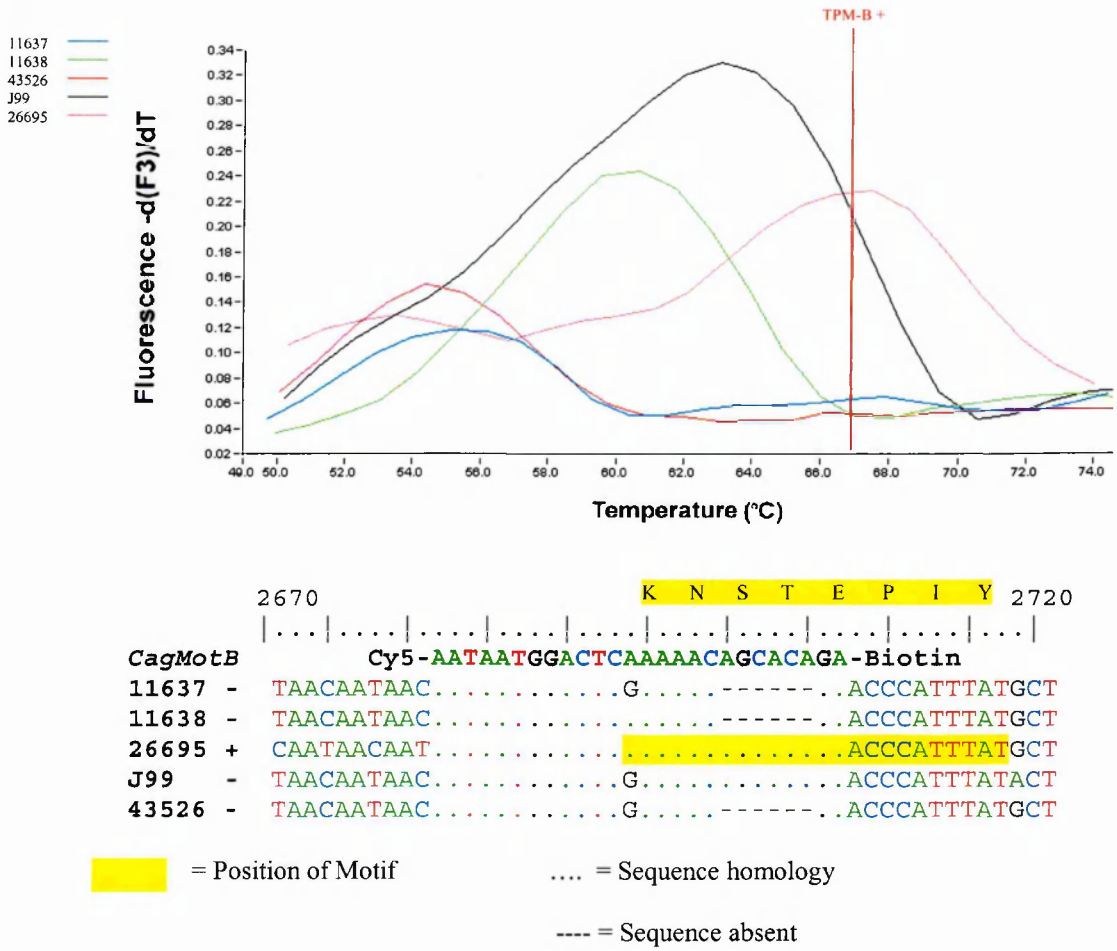



**Figure 7.2: Examples of melting curves generated by five *H. pylori* reference strains, and alignment of *CagMotB* with reference strain sequences.**

Application of the CagMotB assay to the collection of 84 clinical isolates identified TPM-B in three isolates (4 %), all in combination with other motifs. Direct DNA sequencing of two of the positive strains confirmed the presence of TPM-B. For four

other strains that generated Tms similar to that observed for strain J99, DNA sequencing confirmed absence of TPM-B (data not shown).

### 7.2.4 Development and evaluation of *cagA* TPM-C LightCycler assay (CagMotC).

Probe *cagMotC* was designed to match the *cagA* sequence of TPM-C positive reference strains ATCC 43526 and NCTC 11637. Initial testing of reference strains (Appendix A.2.3) by the CagMotC assay demonstrated that these TPM-C positive strains generated peaks corresponding to the highest probe-template Tm (approximately 66.0 °C) (Figure 7.3). The observed Tms for reference strains lacking TPM-C were lower, ranging from 56.0 to 63.0 °C, depending on the level of strain sequence variation (Figure 7.3).

Application of assay CagMotC to 84 clinical isolates identified TPM-C in four isolates, and generated equivocal results in two isolates, as indicated by Tms that were slightly lower than for the positive control strains but higher than for negative control strain NCTC 11638. PCR-RFLP results suggested TPM-C was present in all 6 isolates. Sequence analyses confirmed presence of TPM-C in two isolates that were positive by both assays and in one of the two isolates that generated an equivocal CagMotC assay result (data not shown). PCR-RFLP suggested that TPM-C was present in an additional nine isolates (S. I. Sharp & R. J. Owen, personal communication) but analysis by assay CagMotC did not confirm this, nor did sequencing of five of these. All remaining 70 strains were negative for TPM-C by both CagMotC and PCR-RFLP assays. Overall, TPM-C was detected in 5 strains (6 %) either singly (1 strain) or in combination with other motifs (4 strains). TPM-C was most commonly present in combination with TPM-A (4 strains).

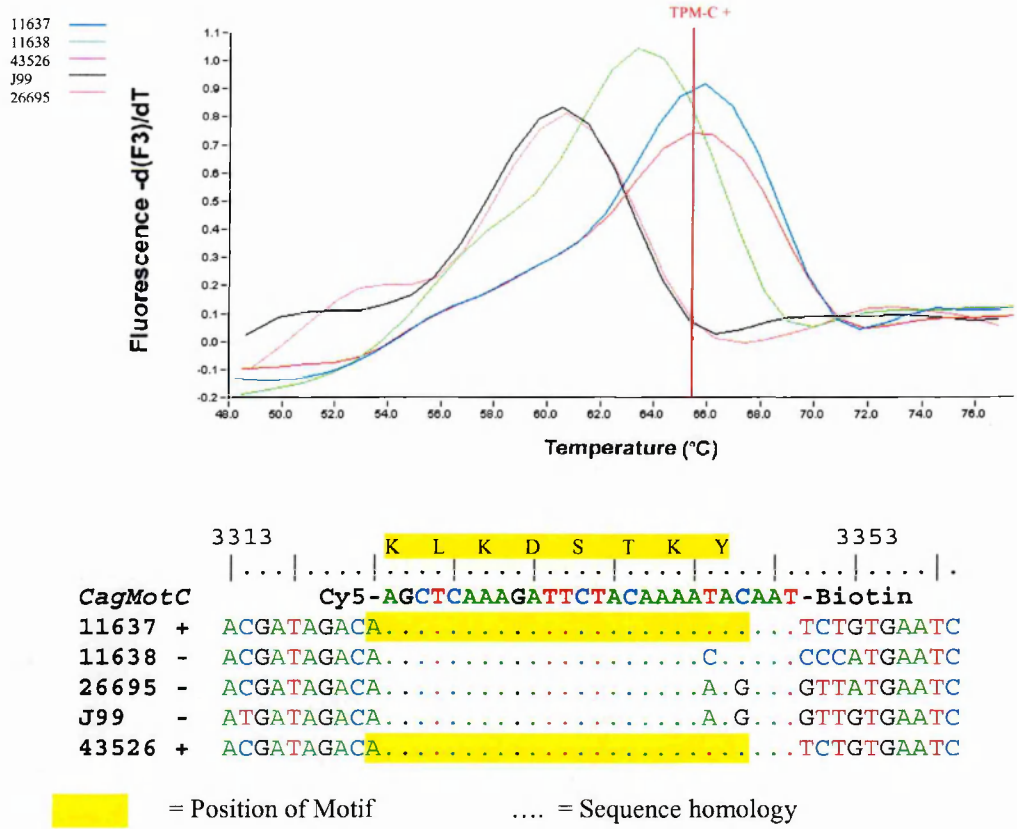



**Figure 7.3: Examples of melting curves generated by five *H. pylori* reference strains, and alignment of *CagMotC* with reference strain sequences.**

### 7.2.5 Determination of *cagA* TPMs directly from gastric biopsies

This study examined a total of 79 gastric biopsies, positive for *H. pylori* by culture and/or histology that were selected from biopsy Groups 4 (n = 17), 5 (n = 39), 6 (n = 18) and 7 (n = 5), defined in Chapter 3. Initial testing of all 79 biopsies by the *cagA* assay described earlier (2.18.2.1) generated specific amplicon in 38 biopsies, 24 of which were from Group 5. Very faint amplicons were observed in ethidium-bromide stained agarose gels in 14/38 cases.

Assays CagMotA, CagMotB and CagMotC were applied to all 24 biopsies that were strongly positive for *cagA* and to five biopsies that had generated faint

amplicons in the initial PCR analysis for *cagA*. The results of these analyses are presented in Table 7.1. The *cagA* TPM-status was determined successfully at all three positions (A, B and C) in 12 samples, all of which were from biopsy Group 5. For the remaining 17 biopsies, at least one of the TPM assays failed to generate a melting peak at the probe hybridisation analysis stage. Probe–template Tms were determined successfully for TPMs A and C only in five biopsies, three of which were TPM-A-positive. Two biopsies generated melting peaks indicative of a *cagA* sequence lacking TPMs B and C, but TPM-A status could not be determined. Melting peaks were generated for assay CagMotC only in the remaining ten samples, all of which lacked that TPM.

**Table 7.1: Determination of *cagA* TPM status directly from gastric biopsies of 29 dyspeptic patients**

| *cagA* TPM | | | | | | Total Number of Biopsies |
|---|---|---|---|---|---|---|
| A | | B | | C | | |
| + | - | + | - | + | - | |
| 9* | 3 | 1* | 11 | 0 | 12 | 12 |
| 3 | 2 | ND† | ND | 0 | 5 | 5 |
| ND | ND | 0 | 2 | 0 | 2 | 2 |
| ND | ND | ND | ND | 0 | 10 | 10 |

*One biopsy was positive for both TPM-A and TPM-B.

†Specific TPM could was not determined by assay CagMotA and/or CagMotB.

For assay CagMotA, the mean cycle number where SYBR Green 1 fluorescence (= amplicon generation) first began exponential increase, termed the crossover threshold ($C_T$) was 23.40 (± 2.21) for biopsies where TPM-A status could be determined, but

was 34.62 (± 2.65) for specimens where the assay failed. This is illustrated in Figure 7.4. Similarly for the CagMotB assay, mean $C_T$ values were higher for specimens where TPM-B status could not be determined (32.67 ± 2.85 *vs.* 25.08 ± 2.58). For both assays CagMotA and CagMotB, these observed differences between $C_T$s of successful and unsuccessful analyses were statistically significant by unpaired *t* test (section 2.25.2), with respective p values of 0.0001 and 0.0002.

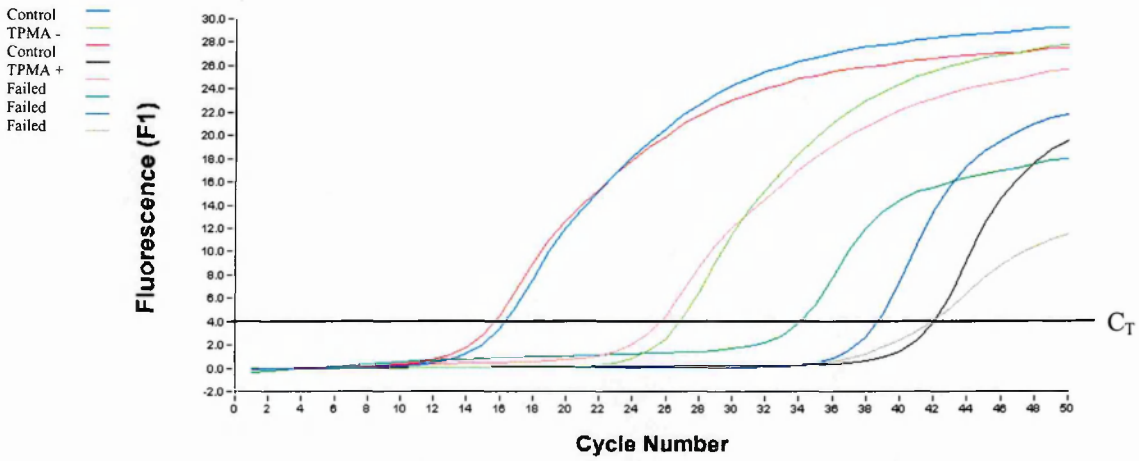



**Figure 7.4: PCR amplification of *cagA* fragments by LightCycler assay CagMotA directly from gastric biopsies.** Amplicon generation, represented by increased SYBR Green 1 fluorescence, occurs earlier for biopsies where TPM-A was successfully determined.

Only the 12 biopsies fully characterised for TPMs A, B and C were included in the examination of relationship between TPM and disease outcome (Table 7.2). As for the bacterial isolates described in section 7.2.1, patients were sub-divided on the basis of endoscopic investigation into either the NUD group (n = 6), the DU group (n = 4) or the GU group (n = 2).

### 7.2.6 Distribution of *cagA* TPMs by disease group.

The distribution of *cagA* TPMs in relation to patient disease is presented in Table 7.2.

**Table 7.2: Distribution of *cagA* TP motifs in 84 *H. pylori* isolates and in 12 gastric biopsies in relation to patient disease.**

| Gastric disease* (n) | *cagA* | | | | | | |
|---|---|---|---|---|---|---|---|
| | No motif | Single motifs | | | Multiple motifs | | |
| | | A | B | C | A, B | A, C | A, B, C |
| GU (16) | 3 | 11 | 0 | 0 | 0 | 2 | 0 |
| DU (27) | 9 | 16 | 0 | 1 | 1 | 0 | 0 |
| GU+DU (4) | 1 | 3 | 0 | 0 | 0 | 0 | 0 |
| GN (2) | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| NUD (47) | 13 | 30 | 0 | 0 | 2 | 1 | 1 |
| Total (96) | 26 | 62 | 0 | 1 | 3 | 3 | 1 |

* GU, Gastric Ulcer; DU, Duodenal Ulcer; GN, Gastric Neoplasia; NUD, Non-Ulcer Dyspepsia.

Overall the *cagA* of 72.9 % of strains contained at least one TPM (Table 7.2). The distribution of the TPMs among clinical isolates and biopsy specimens was slightly higher in GU patients (13/16, 81.3 %) compared to DU patients (18/27, 66.7 %), but this was not statistically significant by Fisher's exact test ($p = 0.484$), described in section 2.25.3. The *cagA* TPM-A was widely distributed amongst isolates and specimens regardless of disease symptoms. In contrast, TPM-B and TPM-C were rare either as a single motif or in combination with TPM-A, and no associations with disease symptoms were evident. Although the prevalence of TPM-A was higher in isolates from GU patients (13/16, 81.3 %) compared to DU patients (17/27, 63.0 %),

again there was no significant correlation between a gastric ulcer–associated strain and presence of that motif ($p = 0.307$). Comparison of the incidence of TPM-A in peptic ulcer disease (PUD) patients (from the GU, DU or GU and DU groups) with that of the NUD group demonstrated no association between PUD and TPM-A ($p = 1.000$). TPM-A was also observed in the two strains associated with GN, but numbers examined were too small to merit statistical analysis.

## 7.3 Discussion

In this study, novel rapid real-time PCR hybridisation assays were developed to test the *H. pylori* genome for the presence of nucleotide sequence motifs that can be used to predict the corresponding deduced three CagA amino acid TPMs (A, B and C) described previously (Odenbreit *et al.* 2000;Odenbreit *et al.* 2001). As these amino acid motifs are proposed to be associated with tyrosine phosphorylation of CagA protein, they were investigated to determine if they might provide novel markers of more severe disease outcome as a result of increased *H. pylori*–host epithelial cell interactions. Polymorphisms within *cagA* may affect the biological function of the protein and might explain the lack of a consistent correlation between *cagA* and disease severity previously noted (Mitchell *et al.* 1996). The original studies on TPMs were based on only a small number of diverse isolates of *H. pylori* so the purpose of this study was to investigate TPM variation and frequency in a larger sample of isolates from peptic ulcer as well as NUD patients.

### 7.3.1 Performances of real-time PCR assays

Three LightCycler probe hybridisation PCR assays (CagMotA, CagMotB and CagMotC) were developed to facilitate rapid, high-throughput detection of each Cag TPM. Initial evaluation of each assay using reference strains of known *cagA*

sequence demonstrated that determination of probe-template Tm distinguished strains containing the relevant TPM from those that did not. Although repeat analyses demonstrated minor inter-run variation of Tms, the relative differences in Tm between different strains remained constant for each run. This highlighted the importance of including appropriate reference strains in each run as controls to allow interpretation of melting peaks generated by unknown samples. Direct DNA sequencing was considered the gold standard to check assay specificities for selected samples but it was not feasible to confirm all results by conventional automated sequencing.

The study population had also been tested for TPMs A and C by three PCR-RFLP assays, two of which were TPM-A specific while the third detected TPM-C. This data was provided by colleagues (S. I. Sharp & R. J. Owen) to establish the relative performances of a real-time PCR versus a conventional block PCR approach. For TPM-A, both approaches successfully classified strains as positive or negative in the majority of cases. However, the identification by PCR-RFLP only of four strains that contained TPM-A demonstrated the potential pitfalls of a probe hybridisation method for identification of specific mutations in a highly diverse gene. Sequence variation anywhere in the 20 - 25 bp region of template complementary to the probe would lower the Tm, even if specific sequence corresponding to the TPM was present. In contrast RFLP was more specific as RE digestion required recognition of very few nucleotides (4 - 6 bp) that were all directly associated with the TPM amino acid sequence. However, the failure of the PCR-RFLP primers to amplify product in one strain that was successfully analysed by the LightCycler-PCR assay again highlights the importance of a multiple primer approach in any PCR-based investigation of a genomically heterogeneous species.

For TPM-C positive strains, LightCycler PCR and sequencing results showed good correlation in most (7/9) cases, although only sequencing could define TPM-C status accurately in the two isolates that had generated equivocal melting peaks by assay CagMotC. In contrast, the PCR-RFLP that used RE *Tsp*509I was less reliable and misidentified TPM-C in nine isolates. It had not been possible to identify a RE specific for the TPM-C sequence, but examination of strain-sequences held in GenBank suggested that the *Tsp*509I recognition sequence found immediately downstream of the TPM-C sequence was only present in strains that contained the C-motif (S. I. Sharp, personal communication). However, results obtained here suggest that although rare, strains that lack TPM-C but contain the *Tsp*509I restriction sequence do occur, and so the PCR-RFLP assay was not sufficiently specific.

In the case of TPM-B, it was only possible to develop a LightCycler assay. Because of the high level of sequence diversity in this region observed in the reference strains, no RE could be found to identify TPM-B by PCR-RFLP analysis (S. I. Sharp, personal communication). The LightCycler assay showed good concordance with direct sequencing, although interpretation was complicated by a number of equivocal results, probably due to the diversity in this region of *cagA*.

One potential limitation of the three LightCycler assays developed in this study was the use of the dye Cy5 to monitor probe-template dissociation. While the wavelength of fluorescence emitted by Cy5 is optimal for the channel settings on the Idaho LightCycler, it is sub-optimal for the settings of the Roche instrument that superseded the Idaho model and was used in this study. It was not possible to adopt the recommended dual-probe format for the Roche LightCycler (described in section 2.20.2.2) as multiple alignment of *cagA* revealed that there was insufficient sequence conservation adjacent to the TPM sites. In contrast, less conservation is necessary for

the bi-probe format that uses a single probe spanning the mutated sequence only. At the time that this study was conducted, Roche had advised that a bi-probe format was not appropriate for this instrument as FRET would not occur between the recommended LightCycler dyes (LC red 640 and LC red 705) and SYBR Green 1. Thus the use of a sub-optimal fluorescent marker (Cy5) in this study may account for some of the difficulties in interpreting melting curves generated, as only a small proportion of the total emitted fluorescence would be detectable by the Roche fluorimeter. In spite of this, results were still unequivocal in most cases. However, assay FS-53 (described in Chapter 10) was developed subsequently and that demonstrated efficient FRET between SYBR Green 1 and LC Red 640. Thus labelling of probes used in assays CagMotA, B or C with appropriate LC Red dyes and assay re-optimisation would be advisable for any future studies of *cagA* TPMs.

Because of the high degree of inter-strain sequence diversity within *cagA*, particularly within the TPM-B region, no single PCR assay format was applicable to all motifs. However, LightCycler TPM assays for motifs A, B and C offer the advantage of simplicity (single tube reaction) and speed, with same-day results generated within one hour, compared to 12-24 h by conventional PCR-RFLP analysis. In addition, the high specificity of PCR-RFLP limits this approach to sequences in a gene that are recognised by an available RE, whereas a probe-based approach is applicable to a wider range of sequences.

### 7.3.2 Determination of *cagA* TPM status directly from gastric biopsies

Initial determination of *cagA* status by conventional block PCR demonstrated that only 48 % of the 79 gastric biopsies tested were positive. Previously, approximately 70 % of *H. pylori* isolates from the same region of South East England had been shown to contain this gene (Owen *et al.* 2001) and the lower prevalence reported in

this study is likely to relate to falsely-negative PCR rather than to true absence of *cagA*. Poor sensitivity of PCR detection assays when applied to gastric biopsies was described in Chapter 3, particularly for one assay targeting *glmM*. The *cagA* assay applied in this study may be similarly insensitive when applied to biopsies where lower target DNA levels exist, possibly due to sample degradation. Evidence that the low *cagA* prevalence is related to specimen quality was provided by the observation that *cagA* was detected in approximately 67 % of the 36 biopsies of Group 5. Sensitivity of *H. pylori* detection by the *glmM* PCR assay had been high in this group also (Chapter 3). It is likely that further optimisation of the *cagA* assay would be necessary to improve sensitivity for the other biopsy groups tested. As the purpose of this analysis was to identify a small number of biopsies that could be tested for *cagA* TPMs, this assay was not optimised further. However, future studies investigating significance of the presence of this gene in relation to differing disease states and other virulence markers, would necessitate assay optimisation for accurate determination of *cagA* status direct from biopsies.

Application of the three LightCycler TPM PCR assays to 29 *cagA*-positive specimens demonstrated that assay CagMotC could determine TPM-C status with high sensitivity (100.0 %), generating melting peaks that were easily interpreted as TPM-C negative in all cases. In contrast, assays CagMotA and CagMotB were less sensitive, with TPM status determined for each in 17/29 and 14/29 biopsies, respectively. All five biopsies that had been defined as weakly positive by conventional *cagA* PCR also failed to generate a result by either of these two assays. For both assays, amplicon was generated in all biopsies. However, analysis of $C_T$ values, that provide quantitative information on specific DNA levels, demonstrated that these were significantly higher in samples that failed to generate a result,

indicating lower initial levels of specific DNA. These observations provide evidence that the most likely explanation for failure of assays CagMotA and CagMotB was that neither was sufficiently sensitive to analyse samples containing lower levels of target DNA. Chapter 8 presents evidence that the LightCycler can detect specific DNA at low levels from stool samples if the assay is first preceded by a round of conventional PCR. A similar nested format approach for the two assays discussed here could overcome the problems of poor sensitivity. This was not attempted for the purpose of this study as it is likely that, for any future investigations, all three TPM assays would be re-designed so that probes were labelled with optimal LC Red dyes, as discussed above. This alteration would maximise fluorescent emissions detectable and may be sufficient to improve sensitivity of probe-hybridisation melting point analysis in these samples. Any sensitivity problems that remained could be addressed at that stage by a nested-PCR format. Nevertheless, the work presented in this chapter demonstrates that characterisation of *cagA* directly from gastric biopsies is possible and highlights the potential for PCR-based analyses of samples where culture is not available. Direct testing of clinical samples allows rapid investigation of a wider study population than would be possible by culture-based studies alone. Real-time PCR assays could provide a powerful strategy for investigating the significance of these putative virulence factors, particularly if used in combination with real-time sequencing approaches such as pyrosequencing that are appropriate for short (25-50 bp) motifs (Monstein *et al.* 2001), to characterise any equivocal results generated by real-time PCR assays.

### 7.3.3 Prevalence of *cagA* TPMs in English isolates and their relationship to disease progression

The results presented in this chapter demonstrate that TPM-A was a common feature (72.9 %) of the 84 *H. pylori* type I strains isolated and the 12 gastric biopsies collected from dyspeptic patients in mid-Essex, irrespective of associations with chronic clinical disease such as presence of gastric and/or duodenal ulcers and gastric cancer. By contrast, TPM-B and TPM-C were less common and detected in only 4.2 % and 5.2 % of strains, respectively, and mostly in combination with TPM-A. Interestingly, the strains with no detectable motif were usually *vacA* m2, which is the strain genotype that interacts least with the host gastric mucosa (Atherton 1998). Although no specific associations between TPM and disease status could be made, it should be appreciated that any study that examines pathogenicity of a chronic infection such as *H. pylori* is limited by a lack of appropriate negative controls. All isolates and gastric biopsies included in this study were recovered from symptomatic (dyspeptic) patients. It is not possible to establish if patients in the NUD group were infected with less virulent strains that were unlikely to lead to further disease or if apparent absence of disease merely reflects early patient investigation and interception of natural progression to a severe clinical outcome.

A recent study investigated TPM frequencies by direct sequencing of appropriate regions of *cagA* in *H. pylori* type I isolates from 15 gastritis and 18 gastric cancer patients in Costa Rica (Occhialini *et al.* 2001). The relative frequencies of two of the TPMs were significantly higher than were found in the study presented here, with 100 % for TPM-A, and 58 % for TPM-B, while TPM-C was not detected in any strain. The reasons for observed differences in both studies is unclear, particularly for motif B, but they could be due to intrinsic differences in the patient populations examined.

Certainly a recent review that surveyed TPM frequencies in *cagA* sequences held in public databases reported a higher TPM-A incidence in isolates of Eastern origin than those from Western countries (Evans, Jr. and Evans 2001). Alternatively the definition of TPM-B in the Costa Rican study as amino acid sequence KNST/GEPIY at position 899 may account for the higher incidence reported (Occhialini *et al.* 2001). Similarly, the higher frequency of TPM-B (58 %) reported from the database survey may be due also to the author's broader definition of potential motifs at this site (Evans, Jr. and Evans 2001). Nevertheless, as observed in this study of English isolates, the Costa Rican study found no association between number of TPMs and the type of disease from which the strain originated. However, the results presented in this chapter suggest a trend, albeit not statistically significant, indicating that a higher proportion of GU-associated compared with DU-associated strains contained TPM-A (81 % *vs.* 63 %). This observation may merit further study on a larger set of such isolates. Analysis of other *cagA* sequences also revealed that only a minority contained TPM-C (Evans, Jr. and Evans 2001). Studies conducted *in vitro* have demonstrated that TPM-C alone was sufficient for tyrosine phosphorylation in AGS cells while TPM-A was not (Puls *et al.* 2002), however the low frequency of this motif suggests that it may not play a role in the pathogenesis of *H. pylori.* In contrast, the higher prevalence TPMs A and B may be more frequently associated with disease. The study by Puls *et al* (2002) also indicated that regions of CagA other than the TPMs identified thus far can lead to tyrosine phosphorylation. Additional TPMs have been identified which are characterised by the peptide sequence EPIYA (Backert *et al.* 2001) that lead to dephosphorylation of host proteins in AGS cells (Stein *et al.* 2002). This amino acid motif is also present as part of TPM-B, and occurs as multiple repeats, located upstream of the TPM-C site at the COOH terminus of CagA. These

TPMs are identical to R1 repeat sequences characterised previously (Yamaoka *et al.* 1998). According to that study, strains which had a CagA containing three EPIYA elements at the C-terminal end, were predominantly isolated from patients with gastric cancer and severe gastric atrophy (Type C), whereas isolates with two EPIYA repeats (Type A) were evenly distributed among patients with different gastric disease symptoms. Further analysis of the C-terminal region showed that both Type A and C strains lacked TPM-C. However, all Type C strains were TPM-B positive whereas approximately a third of the Type A strains lacked TPM-B (S. Rijpkema, personal communication). These findings suggested the presence of TPM-B may also enhance pathogenicity of *H. pylori* whereas the presence of TPM-A in those isolates could not be verified. Thus further investigation of *H. pylori* isolates, particularly of those associated with gastric cancer, is warranted. The remaining 23 isolates in our study did not contain motifs in the PCR assays and in that respect resembled strain J99 (US, duodenal ulcer isolate) although that strain had one R1 repeat 50 bp upstream of the non-functional TPM-C, while the incidence of EPIYA in the study population investigated in this chapter was not determined. Recent evidence suggests that the level of tyrosine phosphorylation, SHP-2 binding and cellular rearrangements relates to the number of EPIYA repeats in Western strains and the arrangement of *cagA* in Eastern strains leads to even greater signal transduction (Higashi *et al.* 2002). Extension of this study to examine this collection of English isolates for motif EPIYA also would allow associations between CagA tyrosine phosphorylation and virulence to be assessed more fully.

## 7.4 Conclusions

The work presented in this chapter has demonstrated that real-time PCR assays developed for the Roche LightCycler are a rapid alternative to sequencing for large-scale screening of *H. pylori* isolates for the specific *cagA* TPMs described previously (Odenbreit *et al.* 2000). Additionally, TPMs can be determined directly from gastric biopsies, facilitating larger investigations of broader populations in the future. The results presented show that while TPM-A is common in patients in mid-Essex there is no evidence in support of a direct association between presence of that TPM or the other rarer TPMs (B and C) and the occurrence of peptic ulcer disease. The presence and disease associations of these and other TPMs such as the EPIYA repeats need to be investigated in a wider selection of strains in relation to the degree of inflammation in the host gastric tissue as well as the level of tyrosine phosphorylation of CagA protein in infected epithelial cells.

# Chapter 8: Comparison of *H. pylori* detection in stool specimens by PCR and by ELISA or immunochromatography kits

## 8.1 Background

The previous chapters have focused on the potential of PCR for detection of helicobacters directly from gastric biopsies. Additionally, the power of PCR as an alternative to culture for gaining additional strain information has been demonstrated in Chapters 5 - 7. One disadvantage of the approaches discussed thus far is that biopsies are collected invasively, posing a small risk to the patient, as well as increasing costs of patient management. Diagnosis of *H. pylori* infection from specimens collected non-invasively would clearly be a preferable alternative. As discussed in section 1.5.2.3, the recent development of two commercial ELISA kits, Premier Platinum HpSA and Amplified IDEIA HpStAR has allowed non-invasive *H. pylori* diagnosis by detection of specific antigen in stools (Vaira *et al.* 2000). Most studies to date have evaluated the former kit only, with a few exceptions that examined the IDEIA HpStAR kit also (Koletzko *et al.* 2003;Leodolter *et al.* 2002;Makristathis *et al.* 2000). These stool tests have been shown to be highly sensitive and specific when applied to adult (Odaka *et al.* 2002;Vaira *et al.* 2002) and to paediatric populations (Konstantopoulos *et al.* 2001;Oderda *et al.* 2000) for testing pre-treatment (Gisbert and Pajares 2001;Konstantopoulos *et al.* 2001;Makristathis *et al.* 2000;Oderda *et al.* 2000) and post-therapy (Gisbert and Pajares 2001;Konstantopoulos *et al.* 2001;Leodolter *et al.* 2002;Makristathis *et al.* 2000;Vaira *et al.* 2002). Additionally, a lateral flow immunochromatography test, ImmunoCard STAT! HpSA is now available in the UK, but no evaluative studies of its performance have yet been reported. PCR-based analysis of stool samples provides an alternative

to stool antigen testing for non-invasive detection of *H. pylori* infection and has the capacity to provide further strain information such as antibiotic susceptibility. However as discussed in section 1.8.2, the sensitivity and specificity of PCR-based detection in studies examining faeces has varied depending on the individual PCR assay and the method of DNA extraction used. Many of the extraction methods have been comparatively complex, requiring multiple steps (Gramley *et al.* 1999;Li *et al.* 1996;Makristathis *et al.* 1998;Monteiro *et al.* 2001a) so it is difficult to envisage the integration of such tests in the present format into a routine laboratory.

The aims of the study presented in this chapter were:

1. To compare relative performances of the two ELISA-based *H. pylori* stool antigen detection tests currently available in the UK in an adult dyspeptic population (pre-treatment) from mid-Essex.
2. To compare performances of *H. pylori* stool antigen detection by ELISA with a rapid immunochromatographic stool antigen test recently launched in the UK.
3. To assess the efficacy of a range of comparatively simple manual and automated methods for DNA extraction from stool specimens artificially seeded with *H. pylori* cells.
4. To evaluate the potential of three nested block cycler PCR assays for detection of *H. pylori* in clinical stool samples.
5. To adapt the nested block cycler PCR assays for detection of *H. pylori* DNA in real-time.

## 8.2 Results

### 8.2.1 Relative performances of ELISA kits for *H. pylori* stool antigen detection

Faecal samples collected pre-treatment from 112 dyspeptic adults aged from 23 – 89 years (mean age 60 years) undergoing routine endoscopic investigation in South East England (as described in section 2.3.2) were tested for stool antigen by two commercial kits following the protocols described (2.23.1 and 2.23.2). Patients were selected on the basis of the results of routine testing of matched patient gastric biopsies by culture and histology at Chelmsford PHL and Broomfield hospital (Chelmsford), respectively. These tests had demonstrated that 64 patients were *H. pylori*-positive by at least one of these methods while no biopsy-based evidence of *H. pylori* infection was found in the remaining 48 patients. The results obtained by the two ELISA kits and the sensitivities and specificities of each test are summarised in Table 8.1, while the quality of the result generated by each assay is illustrated in Figure 8.1. The range of OD readings generated in each patient for the different assays are summarised in Figure 8.2.

The Premier Platinum HpSA kit generated equivocal results in 5 of 64 and 2 of 48 samples from patients that were *H. pylori*-positive and negative, respectively (Table 8.1, Figure 8.2). If equivocal results were defined as stool antigen positive, this raised the assay sensitivity (81.3 %) but lowered the specificity (91.7 %). No equivocal results were generated for the Amplified IDEIA HpStAR kit (Table 8.1, Figure 8.2).

**Table 8.1: Comparison of HpSA and HpStAR stool antigen test performances when applied to 112 stools from patients of known *H. pylori* status**

| Stool Ag kit | | Patient status* (n) | | Sensitivity | Specificity |
|---|---|---|---|---|---|
| Name | Result | + (64) | - (48) | | |
| HpSA | + | 47 | 2 | 73.4 % | 95.8 % |
| | E† | 5 | 2 | | |
| | - | 12 | 44 | | |
| HpStAR | + | 60 | 0 | 93.8 % | 100.0 % |
| | - | 4 | 48 | | |

**H. pylori* status determined by culture and/or histology of gastric biopsies.

†Equivocal results were not included as positive results in calculations of sensitivity and specificity.

As illustrated in Figures 8.1 and 8.2, the Amplified IDEIA HpStAR kit generated results that were easier to interpret than those of the HpSA kit. This is demonstrated also by considering $OD_{450/630}$ values generated for each kit. The mean $OD_{450/630}$ values recorded for HpSA positive and negative specimens were 0.45 ± 0.14 units above and 0.06 ± 0.01 units below the kit's recommended cut-off value for equivocal results of 0.10 OD units, respectively (Figures 8.1 & 8.2). The mean $OD_{450/630}$ values of the IDEIA HpStAR kit for positive and negative results were 1.93 ± 0.34 units above and 0.11 ± 0.01 units below the kit's cut-off level (0.15 OD units) (Figures 8.1 & 8.2). Comparison of means by unpaired *t* test (section 2.25.2) demonstrated that the $OD_{450/630}$ values of the IDEIA HpStAR kit were significantly further from the defined cut-off than that of the HpSA kit ($p = < 0.0001$), and so were easier to interpret.

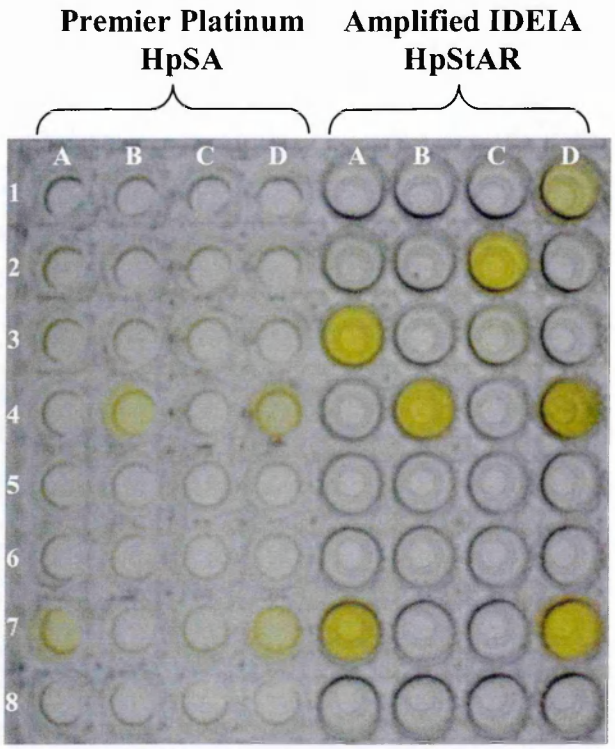
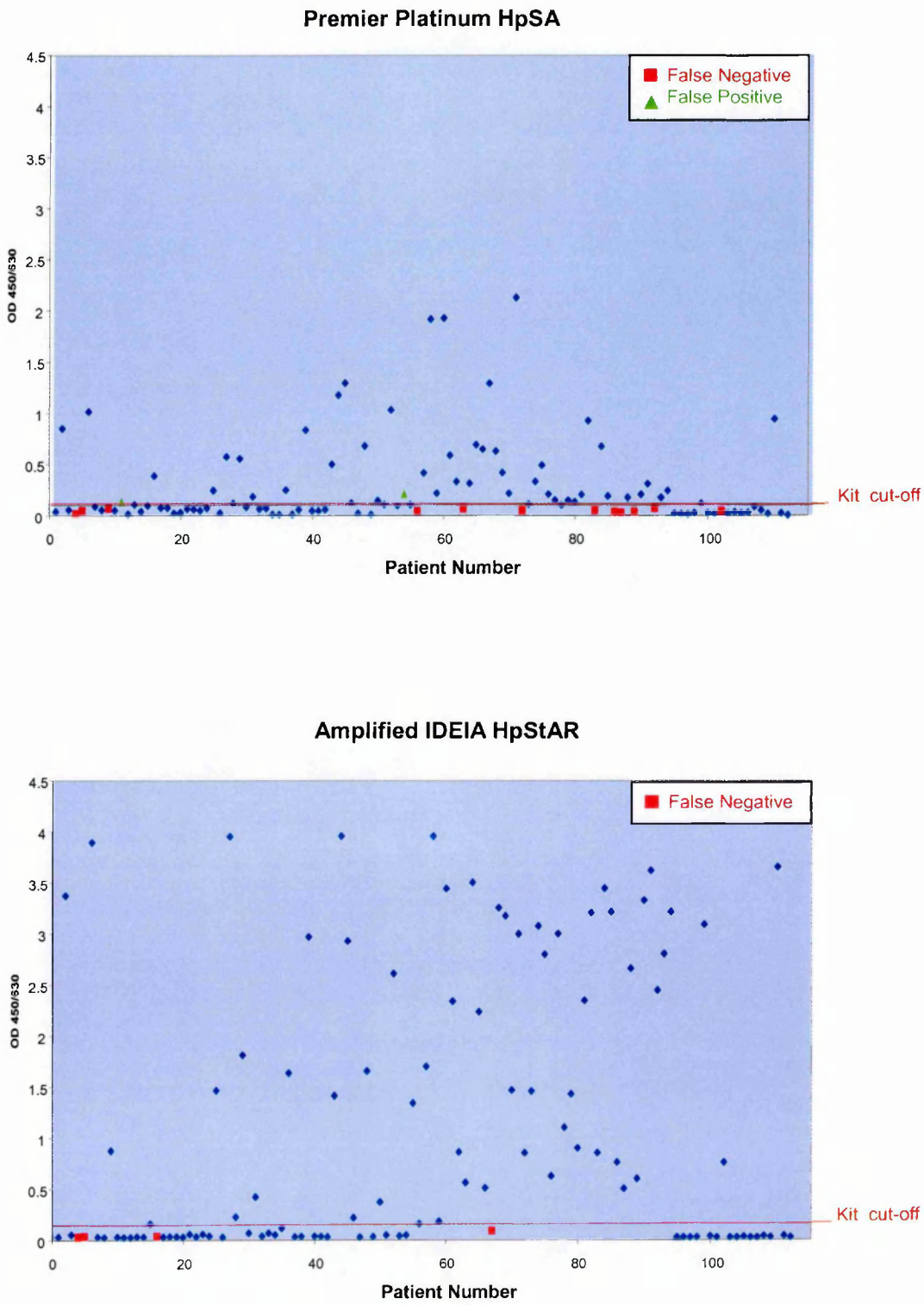



**Figure 8.1: Examples of results generated by the HpSA kit compared with the HpStAR kit.** For both tests, stools from *H. pylori* culture and/or histology positive patients are in wells A2, A3, A7, B4, C2, C3, D1, D4. D7 : kit + control; D8 : kit – control.

**Figure 8.2: Scattergraphs demonstrating the range of $OD_{450/630}$ values recorded for each stool specimen tested by the HpSA kit and by the HpStAR kit.**



### 8.2.2 Comparison of *H. pylori* antigen detection in stools by ImmunoCard STAT! test and HpSA ELISA kit.

Eighty-seven stool samples that had been tested for stool antigen by both ELISA-based kits were examined also by a novel ImmunoCard STAT! HpSA Test as described (2.23.3). Forty-nine of these were from patients that were *H. pylori*-positive by culture and/or histology, while the remaining 38 stools were from *H. pylori*-negative patients. ImmunoCard STAT! test results generated were classified as positive, weakly positive or negative, as illustrated in Figure 8.3.

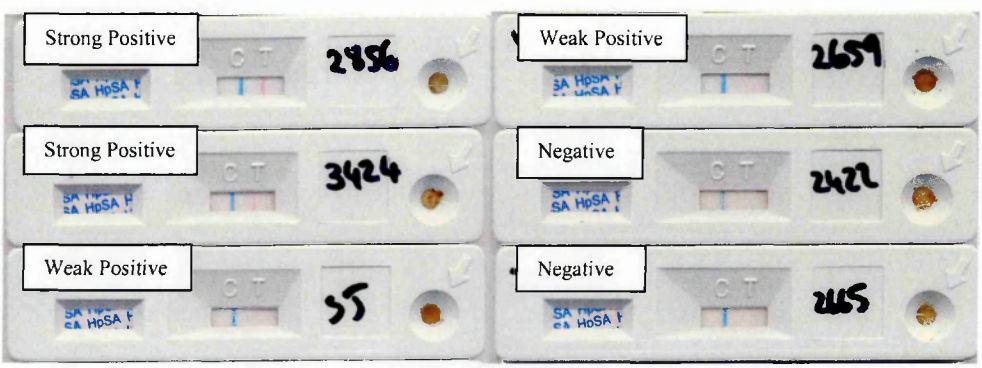



**Figure 8.3: Illustration of *H. pylori antigen* detection in stools by ImmunoCard STAT! HpSA kit.** Strong or moderate positives generated a clear pink band in the test (T) window, while weak positives generated only a faint pink band.

Results generated by the ImmunoCard STAT! HpSA test relative to those obtained for the 87 stools by the Premier Platinum HpSA and by the IDEIA HpStAR ELISA kits are presented in Table 8.2.

**Table 8.2: Results generated by the ImmunoCard STAT! HpSA test when applied to stools from 87 dyspeptic patients, compared with results of the Premier Platinum HpSA ELISA and the IDEIA HpStAR ELISA**

| ImmunoCard STAT! HpSA | HpSA ELISA | | | | | IDEIA HpStAR ELISA | | | |
|---|---|---|---|---|---|---|---|---|---|
| | TP* | TN | E | FN | FP | TP | TN | FN | FP |
| Positive (n = 29) | 25 | 0 | 1 | 3 | 0 | 29 | 0 | 0 | 0 |
| Weak Positive (n = 18) | 8 | 4 | 3 | 3 | 0 | 13 | 4 | 1 | 0 |
| Negative (n = 40) | 2 | 32 | 1 | 4 | 1 | 5 | 34 | 1 | 0 |

*TP = True positive, TN = True negative, E = Equivocal result, FN = False negative FP = False positive, defined on the basis of culture and histology testing of matched gastric biopsies.

Specific antigen was detected by the Premier Platinum HpSA and the IDEIA HpStAR ELISA kits in, respectively, 39/49 and 47/49 *H. pylori*-positive patients and in 2/38 and 0/38 *H. pylori*-negative patients. Positive results were obtained for the ImmunoCard test in 43/49 positive patients and in 4/38 negative patients. The respective sensitivities for the HpSA ELISA, the IDEIA HpStAR and the ImmunoCard tests were thus 79.6 %, 95.9 % and 87.8 %, while test specificities were 94.7 %, 100.0 % and 89.5 %, respectively. However if five equivocal results generated by the HpSA ELISA were considered negative, that test sensitivity was 71.4 % while specificity was 97.3 %.

The stool specimens that were strongly positive by the ImmunoCard STAT! HpSA test had generated mean $OD_{450/630}$ readings of 0.53 ± 0.18 and 2.72 ± 0.33 for the HpSA and HpStAR kits, respectively. In contrast, mean $OD_{450/630}$ readings for stools

that were weakly positive or falsely negative were significantly lower for both the HpSA kit (0.15 ± 0.06, $p = 0.0009$) and the HpStAR kit (0.77 ± 0.34, $p < 0.0001$).

### 8.2.3 Comparison of methods of DNA extraction from stool samples

Initially, faecal samples from *H. pylori*-negative patients were seeded with serial decimal dilutions of bacterial cells to give seven samples containing *H. pylori* concentrations ranging from approximately $10^3$ to $10^9$ cfu/g faeces, as assessed by viable count. DNA was extracted manually from 900-µl aliquots of these by the original and by the modified GuSCN/silica or diatom based methods described earlier (2.12.1), and by a commercial faecal DNA extraction kit (QIAgen Ltd) (2.12.2). Faecal samples (200 µl) were also extracted by the automated MagNAPure system, using the LC Total Nucleic Acid Isolation Kit and the LC DNA Isolation Kit III (Bacteria, Fungi) (sections 2.12.3). All DNA extracts were treated further by dilution or PVP treatment, as described in sections 2.13.1 and 2.13.2. In later experiments, stools were suspended in concentrated milk powder solutions (300 mg/ml) to block non-specific binding of PCR inhibitors to magnetic glass beads during automated DNA extraction.

#### 8.2.3.1 Evaluation of inhibitor removal by DNA extraction methods

All DNA extracts were tested by internal control PCR (2.15.2) to assess the relative abilities of methods to remove PCR-inhibitory substances. Experiments were repeated using stools from different patients, to assess removal of as broad a range of potential PCR inhibitors as possible. The ability of each method to remove inhibitors and assessment of the relative convenience (labour and time) of the methodologies are summarised in Table 8.3.

**Table 8.3: Comparison of efficacy of PCR inhibitor removal and convenience of manual and automated faecal DNA extraction methods.**

| Extraction method | Inhibitor Removal* | | | Ease of use |
|---|---|---|---|---|
| | Post (Pre†) extraction modifications | | | |
| | None (Milk) | 1/10 (Milk) | PVP (Milk) | |
| ***Manual methods*** | | | | |
| GuSCN silica | - | ++ | + | ++ |
| Modified GuSCN silica | + | +++ | ++ | ++ |
| Modified GuSCN diatoms | ++ | +++ | +++ | ++ |
| QIAgen kit | + | +++ | +++ | + |
| ***Automated MagNA Pure methods*** | | | | |
| Total NA isolation kit | - (+) | + (+++) | ++ (+++) | ++++ |
| Bacterial DNA kit III | - (-) | - ‡ (-) | - (+§) | +++ |

*Inhibitor removal represented by the proportion of samples tested that were 16S internal control PCR-positive as follows: - = 0 %; + = < 50%; ++ = 50 – 99%, +++ = 100 %.

†Additional stools suspended in milk solution (300 mg/ml) extracted by automated methods.

‡ DNA extracted by MagNA Pure Bacterial DNA kit III was +++ at 1/100 dilution.

§+++ if PVP-treated extracts amplified by *Tth* instead of *Taq* polymerase in a PCR reaction mix also containing BSA (described in section 2.14.4).

Overall, the modified GuSCN method (containing milk protein in the initial lysis buffer) using diatomaceous particles instead of silica was the most effective of all methods at removing inhibitors, particularly if combined with either dilution or PVP treatment post-extraction. The commercial extraction kit by QIAgen performed

similarly but was more laborious and time consuming. Greater levels of inhibitors remained in the samples extracted by the automated MagNA Pure methods. For the LC Total Nucleic Acid Isolation kit, inhibition could be overcome by initial suspension of stool in milk solution, subsequently combined with either dilution 1/10 or PVP treatment. In contrast, internal control PCR was positive in only some of the DNAs extracted by the Bacterial LC DNA Isolation Kit III unless stool was suspended in milk solution before extraction, the resultant DNA extract then PVP-treated, or diluted 1/100, and BSA included in the final PCR, where, additionally, *Taq* DNA polymerase was substituted with enzyme *Tth*, as described in section 2.14.4.

**8.2.3.2 Evaluation of specific DNA recovery for each DNA extraction method**

DNA extracts were tested by *Helicobacter*- and *H. pylori*-specific PCR assays targeting 16S rRNA and *vacA* (sections 2.16.1.1 and 2.16.3.1), respectively, to assess the relative levels of specific DNA recovery. Generally, the 16S rRNA assay detected lower levels of *H. pylori*, but spiking experiments did not always generate consistent and reproducible results. For this reason, a qualitative summary of specific DNA detection by 16S rRNA PCR for each method is provided in Table 8.4.

Overall, higher yields of DNA, as indicated by PCR detection at lower target gene levels, were obtained for all samples following PVP treatment, compared with dilution as a post-extraction modification. Lowest detection limits were observed when diatomaceous particles were used instead of silica. Lower yields of DNA were obtained for the automated MagNA Pure extraction methods, for both kits tested. Of the limited Bacterial NA DNA extracts tested that were not PCR-inhibitory, only high levels of specific DNA (as much as $10^9$ cfu/ml) could be detected by PCR.

**Table 8.4: Comparison of DNA recovery by various faecal DNA extraction methods, assessed by *Helicobacter*-specific PCR targeting 16S rRNA.**

| Extraction method | | Detection limit* by 16S rRNA PCR | |
|---|---|---|---|
| | | 1/10 | PVP |
| Manual | GuSCN silica | ++ | +++ |
| | Modified GuSCN silica | ++ | +++ |
| | Modified GuSCN diatoms | ++ | ++++ |
| | QIAgen | ++ | +++ |
| Automated | Total NA | + | ++ |
| | Bacterial NA | +/- | +/- |

*Detection limit represented by qualitative scoring system as quantitative data varied between experiments. The lowest level of detection (++++) was $10^4$ cfu/ml, while detection of approximately $10^9$ cfu/ml is represented as +/-.

### 8.2.4 Detection of *H. pylori* in clinical stool samples by PCR

DNA from stool samples of 45 patients were extracted by the modified GuSCN-diatom method (section 2.12.1) and then processed further by the PVP method described previously (section 2.13.2). All 45 samples were positive by the internal control PCR assay described in section 2.15.2. Twenty-three of the 45 patients had been shown to be *H. pylori*-positive by culture and/or histology, while the remaining 22 were *H. pylori*-negative. Sensitivities and specificities of stool antigen tests when applied to these 45 samples were, respectively, 78.3 % and 95.5 % for the HpSA kit and 91.3 % and 100.0 % for the IDEIA HpStAR kit. Preliminary testing of the ten stools from *H. pylori*-positive patients samples demonstrated that single round PCR

assays targeting 16S rRNA and *vacA* (sections 2.16.1.1 and 2.16.3.1) failed to generate specific product. All samples were subsequently tested by three different nested PCR assays amplifying fragments of 16S rDNA, *vacA* and *ahpC,* described in sections 2.16.1.3, 2.16.3.2 and 2.16.5.1, respectively. The sensitivity and specificity of each analysis is summarised in Table 8.5 and examples of the quality of results generated are illustrated in Figure 8.4.

**Table 8.5: Comparison of performances of three nested PCR assays targeting *ahpC*, 16S rRNA and *vacA* genes of *H. pylori* when applied to 45 stools**

| PCR Assay | | Patient status* (n) | | Sensitivity | Specificity |
|---|---|---|---|---|---|
| **Target** | **Result** | **+ (23)** | **- (22)** | | |
| *ahpC* | + | 11 | 3† | 47.8 % | 86.4 % |
| | - | 12 | 19 | | |
| 16S rRNA | + | 8 | 2† | 34.8 % | 90.9 % |
| | - | 15 | 20 | | |
| *vacA* | + | 6 | 1† | 26.0 % | 95.5 % |
| | - | 17 | 21 | | |

*Patient status determined by culture and histology.

†One stool specimen from an *H. pylori*-negative patient was positive by all three PCR assays.

Of the 45 stool specimens examined, 10 were falsely-negative by all three assays. Comparison with matched stool antigen results demonstrated that one of these specimens was also falsely-negative by both antigen detection kits, one was falsely-negative by the HpSA kit only and one had generated an equivocal result by HpSA testing. None of the falsely-positive results generated by PCR analyses were also

positive by stool antigen testing. Overall, the sensitivity and specificity of all three assays combined was 56.5 % and 86.4 %, respectively.

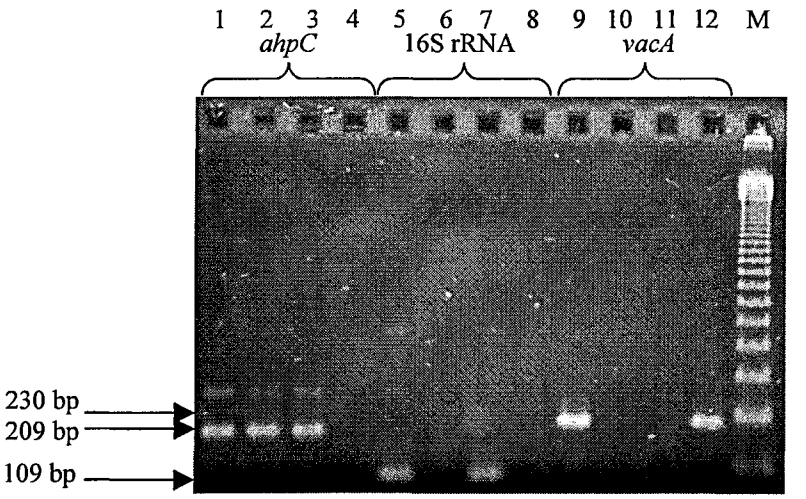



**Figure 8.4: Illustration of PCR analyses of four stool specimens from *H. pylori*-positive patients (A – D) by three nested assays targeting *ahpC*, 16S rRNA and *vacA*.** Patient A (lanes 1, 5 & 9) positive for all three assays. Patient B (lanes 2, 6 & 10) positive for *ahpC* PCR only. Patient C (lanes 3, 7 & 11) positive by *ahpC* and 16S rRNA PCR. Patient D (lanes 4, 8 & 12) positive by *vacA* only. Lane 13, 123 bp marker (M).

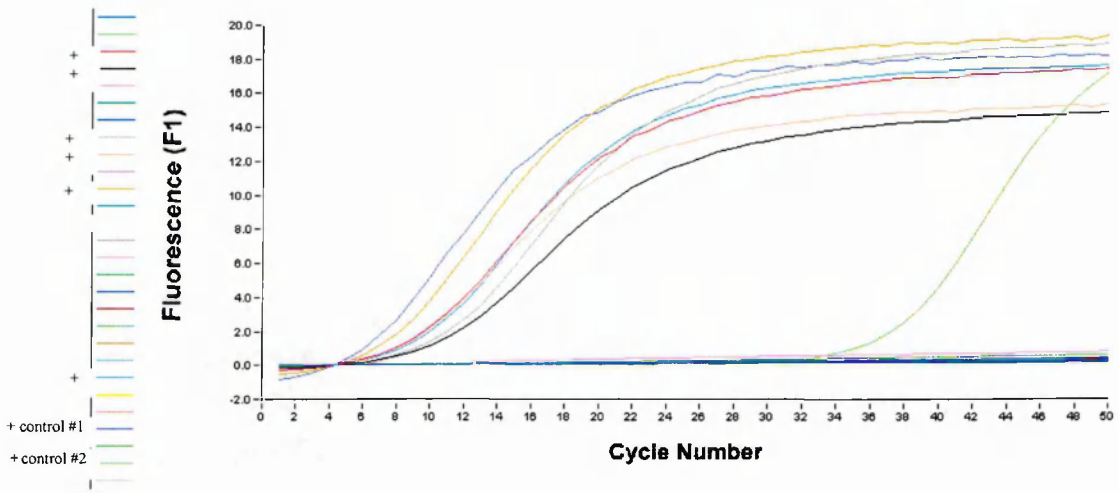
### 8.2.6 Comparison of conventional nested PCR and real-time nested PCR assays

The second round of PCR for both the 16S rRNA and the *ahpC* assays was carried out in real-time on the LightCycler as described (2.20.8 and 2.20.9, respectively) in addition to the conventional PCR analyses described in the section above (8.2.5). Positive results were indicated by an increase in SYBR Green 1 fluorescence (= DNA amplification) as illustrated (Figure 8.5a). Melting peaks generated by monitoring

rate of decrease in SYBR Green 1 fluorescence with increasing temperature were generated in positive samples only (Figure 8.5b). The *ahpC* assay required optimisation of primer concentration to prevent generation of spurious increased fluorescence due to primer dimer formation. Initially, specificity of positive results was confirmed by additional visualisation of amplicons by gel electrophoresis (section 2.19).

Analysis of all 45 stool samples demonstrated that results generated by real-time PCR for both *ahpC* and 16S rRNA assays were identical to those obtained by conventional PCR. Results were easy to interpret, with little evidence of non-specific amplification .

a) PCR amplification




b) Melting point analysis of amplicons

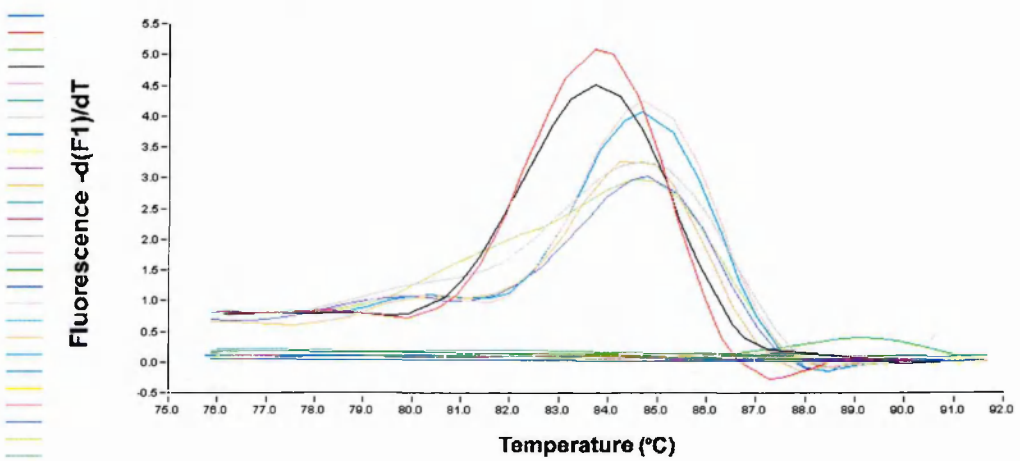



**Figure 8.5: Amplification of *ahpC* in real-time from clinical stool specimens in 2nd round of hemi-nested PCR assay.** a) Positive samples indicated by increased SYBR Green 1 fluorescence. b) SYBR Green 1 melting peaks generated for the positive samples and controls only.

## 8.3 Discussion

The first report of culture of *H. pylori* from stool specimens (Thomas *et al.* 1992) was met with considerable interest as it raised the possibility of an alternative to invasive endoscopy that would not only allow diagnosis of infection but would provide isolates for further characterisation. However, the low success rate of subsequent culture attempts (Dore *et al.* 2000b;Kelly *et al.* 1994) suggested that generally this bile-sensitive organism is non-viable in the lower intestine. With the recent development of immunologically-based tests to detect specific faecal *H. pylori* antigens as well as the potential for PCR-based analysis, stools may still provide an invaluable, non-invasive, means of patient investigation.

### 8.3.1 Comparison of commercial stool *H. pylori* antigen detection ELISA kits

As discussed earlier (section 1.5.2.3), numerous studies have evaluated the potential of the HpSA kit for primary diagnosis of *H. pylori* infection as well as for patient follow-up after eradication therapy. Results from different studies suggested that the sensitivity and specificity of this test may vary according to geographical locations, demonstrating the need for local evaluation of kit performance prior to routine implementation. While one study has examined the performance of the HpSA kit in a paediatric patient group in South West Scotland (Shepherd *et al.* 2000), no other evaluative studies have been conducted in the UK. At the time of writing, comparatively few centres had yet examined the newer Amplified IDEIA HpStAR kit. In this chapter, both kits were evaluated to compare relative performances when applied to stools from dyspeptic adult patients in South East England. The aim of the study was not only to identify the best kit for diagnosis of infection in this geographic location but also to provide direct evidence of *H. pylori* in individual stool samples.

The latter information would facilitate interpretation of the concomitant results of PCR analyses conducted in this study.

Examination of stools from 112 dyspeptic patients demonstrated that while both kits allowed rapid diagnosis of infection (within 1.5 – 2 hours) that was simple to perform, the Amplified IDEIA HpStAR kit was considerably more sensitive and specific. Additionally, this kit was easier to use as washing steps were automated. Results were easier to interpret also, with clearer distinction between positive and negative specimens. In contrast the HpSA kit mean OD for positive results was lower than that of the HpStAR kit while the mean negative OD was higher. These values close to the kit cut off meant that some results could not be interpreted and were defined as equivocal, lowering overall kit sensitivity and specificity.

Given that limited information is available about the precise components of each kit, it is difficult to explain the observed differences in performance. However the monoclonal capture antibodies in the HpStAR kit would allow highly specific binding of *H. pylori* antigen. In contrast, the polyclonal antibodies of the HpSA kit, while potentially more sensitive, could theoretically bind a wider range of faecal antigens non-specifically, leading to higher background OD levels. Use of polyclonal antibodies could also lead to variation in assay performances between kit batches. Additionally, the preliminary centrifugation of diluted stool in the HpStAR kit removes insoluble faecal material, prior to incubation of samples with capture and conjugate antibodies. This may improve accessibility and diffusion of soluble *H. pylori* antigen and so facilitate formation of specific antigen/antibody complexes, leading to higher OD levels for positive results. Removal of solid particles also allowed washing steps to be automated and so standardised between runs. In contrast, the HpSA kit protocol does not include a centrifugation step and both

insoluble and soluble faecal material is incubated with the capture and conjugate antibodies, which may lower the rate of specific complex formation and ultimately OD readings, while increasing non-specific binding and background ODs. Automated well washing was not possible because of the potential for equipment blockage and efficiency of manual washing may have varied between runs. Although care was taken to ensure adequate washing of wells, the amount and nature of insoluble material varied between specimens and so too would the levels of residual material that may have contributed also to the high non-specific background ODs observed in some samples. Of the two ELISA-based kits tested for *H. pylori* stool antigen detection, the IDEIA HpStAR kit is apparently preferable for pre-treatment *H. pylori* diagnosis in adult dyspeptic patients in South East England.

Few studies have compared the relative performances of these two kits (Koletzko *et al.* 2003;Leodolter *et al.* 2002;Makristathis *et al.* 2000). The findings of this study of an adult dyspeptic population were in agreement with those of an earlier study of paediatric patients that demonstrated marginally higher sensitivity (98.0 %) of the IDEIA HpStAR (= FemtoLab) kit for primary diagnosis of *H. pylori* infection, compared with the HpSA kit (93.8 %) (Makristathis *et al.* 2000). Additionally, that study demonstrated that both kits were suitable for patient follow-up post therapy. A subsequent study of 148 adult patients reported marginally higher sensitivity of the HpStAR kit for evaluating success of eradication therapy also, although the kit cut-off OD was lowered to 0.90 to achieve this (Leodolter *et al.* 2002). None of the patients reported on in this chapter had received specific eradication therapy, but extension of this study to include such patients would provide information on the utility of these tests for patient- follow-up, and allow appropriate cut-offs to be established for this

application. Likewise, examination of paediatric populations will be essential to fully evaluate the performances of these kits in patients from the UK.

### 8.3.2 Detection of *H. pylori* antigen in stools by ImmunoCard STAT! test

Both ELISA-based kits required inclusion of a positive and a negative control in each run. It was therefore most economical to batch-test multiple stool specimens in a single experiment. In contrast, the immunochromatographical ImmunoCard STAT! HpSA test is more suitable for testing single or small numbers of stools. This test uses monoclonal antibodies to capture specific antigen, and results can be obtained in 5 – 10 minutes, compared with 2 – 3 hours with ELISA-based methods. Analysis of 87 stools demonstrated that stools that had generated high OD readings by ELISA also generated clear positive results for this test. However, weak positive results or falsely negative results corresponded to low OD readings by ELISA and were very difficult to interpret. This speed and simplicity of this test is clearly advantageous compared with ELISA, particularly in a laboratory that examines low numbers of stool specimens. High sensitivity and specificity has been reported for similar ImmunoCard tests available for detection of enteric pathogens *E. coli* O157 (Mackenzie *et al.* 2000) and *Giardia lamblia* and *Cryptosporidium parvum* (Garcia *et al.* 2003). To date, no studies evaluating the performance of this test for *H. pylori* detection have been published. Results presented in this chapter suggest that the test was more sensitive, but less specific, than the HpSA ELISA and allows accurate detection of *H. pylori* from stools with higher levels of antigen. However, weakly positive results were defined subjectively and as for the equivocal results of HpSA ELISA, would be difficult to interpret in a clinical setting. Thus while this test is more convenient than ELISA, the higher sensitivity and specificity of the IDEIA HpStAR test suggests that this should be the test of choice for *H. pylori* antigen

detection from stools. Examination of greater numbers of stools recovered before and after treatment of patients will be necessary to fully evaluate the performance of this test.

### 8.3.3 Comparison of manual and automated methods of stool DNA extraction

Stool antigen detection allows sensitive and specific detection of *H. pylori*. However, unlike PCR, it does not have the potential to provide any further strain information. Various strategies have been applied to extract *H. pylori* DNA from stool specimens, including immunomagnetic separation (IMS), polypropylene filtration, phenol chloroform extraction, commercial GuSCN-based kits and use of specific DNA capture probes (Gramley *et al.* 1999;Li *et al.* 1996;Monteiro *et al.* 2001b;Monteiro *et al.* 2001a;Parsonnet *et al.* 1999;Russo *et al.* 1999;Shuber *et al.* 2002;Watanabe *et al.* 1998). However as many of the described methods appeared to be complex, time-consuming and laborious, it seems unlikely that they could be introduced into a routine diagnostic laboratory.

This study aimed to establish an effective extraction method that was rapid and comparatively simple, to reduce the risk of introduction of exogenous DNA and also to maximise its potential for application in routine diagnostic laboratories. Development of simpler extraction methods suitable for routine use could potentially revolutionise management of patients currently investigated non-invasively, as it would facilitate extensive PCR-based analyses that could allow further strain characterisation.

Internal control PCR demonstrated that inhibitory substances remained for extracts prepared by all methods examined. For the manual methods, PVP treatment or 1/10 dilution was sufficient to remove inhibitors from DNA of all stools extracted by the commercial QIAgen kit. The in-house GuSCN-based method was significantly

improved by inclusion of milk protein in the first GuSCN buffer, to block binding of inhibitors, and the use of diatoms, that pellet less tightly than silica and so can be washed more thoroughly. Assessment of DNA recovery by *Helicobacter*-specific PCR demonstrated that detection limits varied between different seeding experiments, highlighting a limitation of this artificial approach. Faecal samples were difficult to homogenise sufficiently to ensure identical composition of aliquots removed for extraction from each dilution. Unequal proportions of soluble to insoluble material would alter the levels of specific DNA removed for extraction, leading to variation in detection limits obtained. Nevertheless, the experiments conducted allowed qualitative assessment of relative DNA yields for each method. Although the modified GuSCN-diatom method was only marginally superior in terms of specific DNA yield, it was less laborious than the commercial kit and was one third of the cost (estimated reagent costs per extraction £ 0.90 *vs.* £ 2.75). Of the two post-extraction treatments, PVP is preferable as it concentrates the DNA extract whereas dilution of specific target would lower sensitivity.

Although simple, the modified GuSCN-diatom method is not high-throughput, with an estimated maximum of 30 – 40 extractions possible per day. In contrast, the MagNA Pure offers simple "walk away" DNA extraction of 32 samples in approximately 2 hours. Preliminary experiments demonstrated that DNA extracted by either the MagNA Pure LC Total Nucleic Acid Isolation Kit or the LC DNA Isolation Kit III (Bacteria, Fungi) contained higher levels of residual inhibitors than those extracted by manual methods. Inclusion of milk protein in the initial sample diluent and post-extraction PVP treatment was sufficient to remove inhibitors from the LC Total Nucleic Acid Isolation Kit extracts. In contrast, these plus additional modifications of the PCR reaction (using BSA and *Tth* polymerase) were necessary to

remove the higher levels of inhibitors remaining in the LC DNA Isolation Kit III (Bacteria, Fungi) extracts. Furthermore, specific DNA recovery for both kits was substantially lower than that of the manual GuSCN-diatom method. The MagNA Pure system was recently reported to successfully extract DNA from stools for PCR-based diagnosis of microsporidia infection (Wolk 2002). The results presented here demonstrate that problems of residual PCR inhibitors in DNA extracted by this system can be overcome by a series of relatively simple modifications. However given the culture and PCR-based evidence to suggest that *H. pylori* levels are low in the colon, the poor recovery of specific DNA with the MagNA Pure make it unsuitable for sensitive *H. pylori* detection. For these reasons, the modified GuSCN-diatom extraction method combined with PVP treatment was applied in subsequent analyses of clinical stool specimens. Since this study was conducted, S.T.A.R. (Stool Transport and Recovery) Buffer has been developed that is reported by the manufacturers (Roche Diagnostics Ltd) to inactivate nucleases, bind inhibitors and stabilise nucleic acid. As this buffer is compatible with the MagNA Pure system, future studies should be conducted to establish if this could overcome the problems discussed here.

### 8.3.4 Detection of *H. pylori* from clinical stool specimens by conventional PCR

Preliminary analyses of stool DNA extracts confirmed the results of earlier studies that single-round PCR was insufficiently sensitive for *H. pylori* detection from stools. Given that the large intestine is not the natural niche for *H. pylori*, it is likely that comparatively few organisms are present in stools compared with the gastric mucosa. It is therefore not unexpected that the PCR assays that allowed sensitive detection of *H. pylori* in gastric biopsies in Chapter 3 perform differently in these samples. PCR

assay formats (nested and hemi-nested) involving two rounds of amplification were applied to improve sensitivity of detection.

Three such nested PCR assays were investigated, one targeting *ahpC* (= 26 kDa specific antigen gene), that had previously been shown be highly sensitive and specific (Makristathis *et al.* 1998;Makristathis *et al.* 2000) and two novel assays targeting 16S rRNA and *vacA* genes. The strategy for the 16S rDNA assay was firstly to amplify all species of *Helicobacter* DNA present in stools and then to amplify only fragments of the *H. pylori* genome in the second round. Other helicobacters have been identified in human stools (Fox 2002;O'Rourke *et al.* 2001) including *H. pullorum* that may be associated with diarrhoeal disease (Burnens *et al.* 1994;Stanley *et al.* 1994;Steinbrueckner *et al.* 1997). Higher levels of other helicobacters could generate positive results after the first round of PCR that would not be apparent in the second *H. pylori*-specific PCR. Therefore, this novel approach could have the potential to not only detect *H. pylori* infection but also to provide a means of surveillance of other helicobacters in the human large intestine, although no examples of this were found in this study. As the levels of prokaryotic 16S rDNA are extremely high in stool specimens, assay primers could cross-react with other bacterial species and lower specificity. Likewise, *ahpC* homologues have been shown to be present in other helicobacters (Lundstrom *et al.* 2001) that may be present in human stools samples. By contrast, *vacA* is found only in *H. pylori* and so a third nested assay, based on the novel primers designed for the HpVac assay, was designed and evaluated.

Application of the three PCR assays to 45 faecal DNAs extracted by the modified GuSCN-diatom plus PVP method demonstrated that the *ahpC* assay was the most sensitive but least specific while the *vacA* assay was considerably less sensitive but

was the most specific. Overall sensitivity of *H. pylori* detection from stools by the three PCR assays combined was 56.5 %, while specificity was 86.4 %. In contrast, ELISA-based testing of stools specific antigen, particularly by the IDEIA HpStAR kit was a more sensitive (91.3 %) and specific (100.0 %) means of *H. pylori* detection in the same stool samples.

As discussed earlier (section 1.8.2), success of PCR-based detection of *H. pylori* in stools has been variable, with wide ranges of sensitivities reported (Gramley *et al.* 1999;Li *et al.* 1996;Makristathis *et al.* 1998;Makristathis *et al.* 2000;Mapstone *et al.* 1993b;Monteiro *et al.* 2001a;Monteiro *et al.* 2001b;Parsonnet *et al.* 1999;Russo *et al.* 1999;Shuber *et al.* 2002;van Zwet *et al.* 1994;Watanabe *et al.* 1998). However differences in study populations, specimen storage and extraction methods as well as the PCR assay applied complicate comparisons between studies. Likewise it is difficult to establish if poor sensitivity is due to absence of specific DNA in specimens or due to an inadequacy of the methodologies applied. Stool antigen testing provided direct evidence that 20 stools included for PCR analysis in this study contained *H. pylori* antigen. As stools were frozen immediately on receipt at the primary laboratory (Chelmsford PHL), culture was not attempted and so it is not known if these were intact cells or fragments of *H. pylori.* The limited success of culture in other studies suggest that the latter is more likely. The observed higher sensitivity for the ELISA kits could therefore be explained by greater stability of the specific antigen, compared with that of specific DNA. Results presented in Chapter 3 suggested that *H. pylori* DNA may be relatively labile and subject to degradation. Stool samples contain many thousands of bacterial species and so numerous DNAases. Specific DNA degradation may be an even greater problem in stool specimens than in gastric biopsies. Differences between ELISA and PCR sensitivities

could be partially attributable to sampling errors also, as 100 µl of diluted stool is examined by ELISA compared with 5 µl DNA extract for PCR. This may influence test outcome considerably, particularly if only low levels of *H. pylori* are present in stool specimens.

One study that examined stools collected pre-treatment from 63 *H. pylori*-positive and 37 negative patients demonstrated that the same *ahpC* hemi-nested PCR assay evaluated in this chapter was more sensitive than the HpSA kit (93.7 % *vs.* 88.9 %) in an adult dyspeptic population (Makristathis *et al.* 1998) as well as being more specific (100.0 % *vs.* 94.3 %). Similarly high sensitivity was reported in a subsequent study examining stools from 49 paediatric patients (Makristathis *et al.* 2000). Although the *ahpC* assay was the most sensitive of the three PCR assays examined in this chapter, overall sensitivity was lower than reported previously by Makristathis. In those two earlier studies, DNA was extracted by suspension of stools in lysis buffer, centrifugation, supernatant boiling, further centrifugation and then RNAse and proteinase K digestion of the supernatant. This was followed by addition of GuSCN and column chromatography, three purifications of the eluate with organic solvents and a final concentration by filtration. This approach may have allowed superior recovery of specific DNA and so higher PCR sensitivity. However the method is so complex, time-consuming and laborious that it seems unlikely that it could be applied in a routine diagnostic laboratory. Alternatively, the observed differences in assay performance could be due to differences in study populations or conditions of specimen storage. In the current study, patients were requested to bring a stool specimen collected the same day as endoscopy for submission at that time, but the actual time between specimen collection and presentation to the endoscopy clinic was not known. Prolonged incubation of stools at room temperature may have resulted in

specific DNA degradation in some samples, lowering PCR sensitivity. Additionally, as fewer patients were examined in the current study compared with those conducted previously, precise comparisons between studies are difficult. Extension of the current study to examine a larger population would allow more accurate assessment of assay performance.

### 8.3.5 Detection of *H. pylori* in stools by real-time PCR

One disadvantage of nested PCR is that the second round of amplification substantially increases the overall test time, to about 7 - 8 hours, including gel electrophoresis. In this study, the second PCR for the *ahpC* and the 16S rRNA assays was modified for the LightCycler, so that nested amplicon could be generated in real time (approximately 40 minutes, compared with 2 – 3 hr with the block cycler PCR), giving a total test time of approximately 4 hours. These LightCycler assays were equally sensitive and specific, compared with the conventional block approach, and were easy to interpret. This development could significantly improve total specimen turnaround time as not only is the second PCR round considerably faster but subsequent gel electrophoresis is not required. Combination of the simple extraction methods along with this nested real-time PCR approach allows *H. pylori* detection within 36 hours of initial specimen receipt. This is the first description of *H. pylori* detection from stool specimens by real-time PCR.

As for the conventional nested PCR assays, further optimisation of parameters including specimen transport and storage, DNA extraction methods and PCR assays will be necessary to improve sensitivity of detection. Nevertheless, the work presented in this chapter demonstrates the potential for examination of *H. pylori* infections non-invasively by PCR, using comparatively simple methods. This approach could be most valuable as a tool for investigating patients currently not

undergoing endoscopy and culture, particularly as PCR provides a means of further strain characterisation, as demonstrated in Chapters 5 – 7. As discussed in Chapter 5, determination of antibiotic susceptibility is particularly important as this can have a significant impact on patient management and eradication therapy outcome.

As all CLA susceptibility assays described thus far (section 1.11.3) involve only one round of PCR, they are unlikely to be sufficiently sensitive. Molecular CLA susceptibility tests therefore could not be applied to stool specimens in their present format. Future modification of existing assays to a nested format could address potential problems of specificity and sensitivity and allow CLA susceptibility testing directly from stools. For instance, development of *H. pylori*-specific primers to amplify specific fragments of 23S rDNA, followed by a second round of PCR with internal primers to amplify the region of 23S rDNA for identification of mutations associated with CLA resistance. It has been demonstrated in this chapter that nested PCR from stools using the LightCycler is possible, so it is likely that a similar approach using a modification of the assay described in Chapter 5 could allow direct determination of CLA susceptibilities from stools.

## 8.4 Conclusions

The IDEIA HpStAR kit for specific antigen detection is a sensitive and specific test for non-invasive diagnosis of *H. pylori* infection in dyspeptic patients from South East England. In contrast, results generated by both the Premier Platinum HpSA kit and the ImmunoCard STAT! test were more difficult to interpret. Although automated DNA extraction by the MagNAPure system would allow high-throughput specimen processing, the lower yields of DNA obtained suggest that this is unlikely to allow sensitive detection of *H. pylori* from stools, compared with manual extraction

methods. PCR-based detection *of H. pylori* from stools was less sensitive and specific than antigen detection tests, but that approach is unique as it could be developed to provide additional strain information from samples collected non-invasively. Additionally, it has been shown for the first time that real-time PCR based detection of *H. pylori* in stools is possible without any loss in assay sensitivity or specificity, compared with conventional block PCR.

# Chapter 9: PCR-based detection of *Helicobacter* species at extra-gastric sites.

## 9.1 Background

The previous chapters have focused on the use of PCR to investigate *Helicobacter* species associated with gastric disease. However PCR has also provided a powerful tool for investigating associations between *Helicobacter* infection and extra-gastric diseases, for example in the hepatobiliary tract (Fox *et al.* 1998;Lin *et al.* 1995;Metz 1998;Nilsson *et al.* 1999;Roe *et al.* 1999), the cardiovascular system (Blasi *et al.* 1996;Danesh 1998) and the lower gastrointestinal tract (Fanning *et al.* 1998;Tiveljung *et al.* 1999). In the present chapter, PCR was applied to bladder, colonic and bronchial biopsies to investigate the possible involvement of helicobacters, by virtue of their association with chronic inflammatory disease, in four chronic inflammatory conditions with poorly understood aetiology, namely:

**Interstitial cystitis (IC)**, a condition characterised by chronic mucosal inflammation of the bladder, often accompanied by ulceration. This syndrome is analogous to *H. pylori* induced inflammation of the gastric mucosa (Warren 1994). *H. pylori* can cause inflammation of the murine urinary tract (Isogai *et al.* 1994) while in humans, *H. pylori* eradication therapy has been reported to improve IC symptoms (English *et al.* 1998).

**Crohn's disease (CD)** and **ulcerative colitis (UC)**. Both conditions are forms of inflammatory bowel disease (IBD), and are thought to result either from an abnormal reaction to the constant antigenic stimulation provided by commensal bacteria in the lower GI tract, or alternatively to be attributable to specific, as yet unidentified, bacterial or viral infections. PCR and probe hybridisation-based studies have suggested the involvement of mycobacterial species, particularly in CD (Hermon-

Taylor 2000;O'Mahony and Hill 2002;Roholl *et al.* 2002;Ryan *et al.* 2002); however this association is still not universally accepted (el Zaatari *et al.* 2001). Interestingly, serological studies have indicated an inverse relationship between *H. pylori* infection and IBD symptoms (Halme *et al.* 1996;Kolho *et al.* 1998;Parente *et al.* 1997a;Pearce *et al.* 2000), whereas two PCR-based studies reported helicobacter DNA from the colon of CD patients (Tiveljung *et al.* 1999;Bohr *et al*, 2002).

**Chronic persistent cough (CPC)** describes a broad category with a wide range of recognised causes in many patients (e.g. smoking, asthma), but with no definitive aetiological agent identified. CPC does not respond to conventional antibiotic therapies aimed at respiratory pathogens and is often observed in patients with gastro-oesophageal reflux disease (GERD) (Philp 1997). As discussed in sections 1.8.1 and 1.9.1, *H. pylori* DNA has been detected in various sites of the oral cavity (Berroteran *et al.* 2002;Li *et al.* 1996;Mravak-Stipetic *et al.* 1998;Riggio *et al.* 2000), and *H. pylori* has been recovered from tracheobronchial aspirates of mechanically ventilated patients (Mitz and Farber 1993). Additionally, serological studies have suggested higher incidences of *H. pylori* infection in patients with chronic bronchitis (Caselli *et al.* 1999;Roussos *et al.* 2002) and with bronchiectasis (Tsang *et al.* 1998). To date, no studies have examined lung tissue from CPC patients for helicobacters.

The aims of the work presented in this chapter were:

1. To investigate for the first time, potential associations between helicobacters and inflammatory conditions of the bladder, colon and bronchi using multiple PCR assays specific for the *Helicobacter* genus, *H. pylori* and HHLOs.

2. To characterise amplicons from biopsy tissues by sequence and BLASTn analyses.

## 9.2 Results

### 9.2.1 Study populations

Bladder biopsies, colonic biopsies and bronchial biopsies were collected and stored as described in sections 2.3.3, 2.3.4 and 2.3.5, respectively.

Bladder biopsies were collected from the right and left bladder wall of 27 patients (A – $Z_2$). Ten of these (patients F, G, J, K, L, M, N, P, T, U) had symptoms consistent with IC, as defined by the U.S. National Institute of Health guidelines (F.H. Anjum, personal communication). The remaining 17 patients, included as a control group, had other urological symptoms (e.g. haematuria and incontinence). Work conducted in Chelmsford PHL demonstrated that bladder biopsies and urine samples were culture-negative (for helicobacters and other bacterial species), while one IC patient (F) and one control patient (R) were *H. pylori*-seropositive.

Colonic biopsies were collected from 30 patients who had either CD (9), UC (11) or were control patients (10) with other symptoms including rectal bleeding, intestinal polyps, diarrhoea and constipation. Routine histological examination of biopsies at St. Mark's Hospital did not demonstrate presence of helicobacters.

Bronchial biopsies were collected from 10 patients with CPC. Routine investigations for respiratory pathogens carried out at Taunton PHL had failed to determine a conventional cause for this condition in these patients.

### 9.2.2 Internal control PCR

DNA was extracted from bladder and colonic biopsies by the method described (section 2.11.2.1) and evaluated for gastric biopsies in Chapter 3. Visual assessment of bronchial biopsies suggested that these contained significantly higher levels of blood than all other biopsies investigated in this thesis, so these were extracted by a commercial kit (section 2.11.3) developed for DNA extraction from blood and vascular tissue. All biopsy extracts were tested by the internal control PCR described in section 2.15.1 to ensure that PCR inhibitors did not remain following either method of DNA extraction. The expected 823-bp amplicon of human cytochrome oxidase DNA was generated in all biopsies.

### 9.2.3 PCR-based detection of helicobacters in bladder biopsies

All bladder biopsies were analysed by the two *Helicobacter* genus-specific assays described in section 2.16.1.1, by the *H. pylori* specific assays targeting 16S rRNA and *vacA* genes described in sections 2.16.1.2 and 2.16.3.1, and also by the HHLO specific assay described in section 2.16.1.4. Amplicons were generated by at least one assay in 23/27 (85.2 %) biopsies and by two or more assays in 5/27 (18.5 %) specimens. Biopsies were PCR-positive by at least one assay in 9/10 (90.0 %) of the IC patients and in 14/17 (90.0 %) of the control group. Positive results were obtained by two or more PCR assays in 3/10 (30.0 %) of the IC group and 2/17 (11.8 %) of the control group, although these differences were not statistically significant ($p = 0.3261$) when analysed by Fisher's exact test (section 2.25.3). All biopsies were negative by the HHLO-specific assay. Retrospective application of the prokaryote-specific PCR assay of Fox *et al* (1995), described in section 2.16.1.1 generated amplicon in 11/27 patients. The different combinations of PCR-positive results

obtained are presented in Table 9.1 and examples of band intensities generated by each PCR assay are provided in Figure 9.1.

**Table 9.1: The different combinations of results generated by five PCR assays when applied to human bladder biopsies from 27 urology patients**

| PCR assay | | | | | Number of Patients | Patient Code |
|---|---|---|---|---|---|---|
| | *Helicobacter* specific | | *H. pylori* specific | | | |
| Fox*† | Bohr* | Logan* | Ho* | HpVac | | |
| + | + | + | + | + | 1 | B |
| - | + | + | + | + | 1 | M |
| + | + | + | + | - | 2 | A, F |
| + | + | + | - | - | 1 | G |
| + | - | + | - | - | 7 | C - E, H - J, L |
| - | + | - | - | - | 11 | N, P, Q - X, $Z_2$ |
| - | - | - | - | - | 4 | K, O, Y, $Z_1$ |

*PCR assays that target 16S rRNA genes (illustrated in Figure 1.3).

†Prokaryote-specific PCR assay.

+ = PCR-positive.

- = PCR-negative.

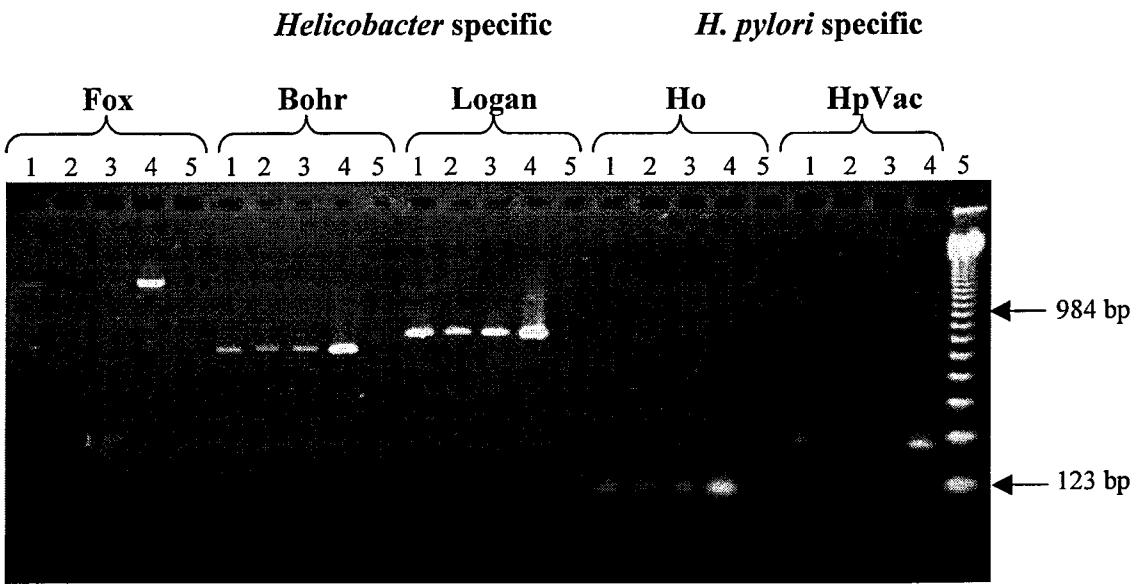



**Figure 9.1: Examples of prokaryote-specific, *Helicobacter* genus and *H. pylori* specific PCR positive results generated from bladder biopsies.** For each PCR assay, reaction order from lane 1 – 5 in each section is patient B (positive for all assays), patient M (positive for all assays but that of Fox *et al* (1995), patient A (positive for all assays but HpVac), positive control, negative control.

### 9.2.4 Identification of PCR products amplified from bladder biopsies

For those biopsies that generated adequate levels of product for further analysis (Figure 9.1), detection assay amplicons were sequenced as described in section 2.22. A total of 18 amplicons were sequenced, comprising 16 fragments of 16S rDNA generated by assays developed by Fox *et al* (1995) (n = 2), Logan *et al* (2000) (n = 8), Bohr *et al* (1998) (n = 5) (section 2.16.1.1) and Ho *et al* (1992) (n = 1) (section 2.16.1.2) and also two *vacA* fragments generated by the HpVac assay (section 2.16.3.1). Subsequent alignment of 16S rDNA sequences with published sequences

held in public databases (BLASTn analysis) demonstrated that these were most similar (> 99 %) to *H. pylori* sequences while *vacA* sequences were up to 97 % similar to those held in GenBank. Similarity of 16S rRNA sequences of PCR products generated by the assay of Logan *et al* (2000) compared with selected 16S rRNA sequences of a range of 17 species of *Helicobacter* available in GenBank is represented in Figure 9.2.

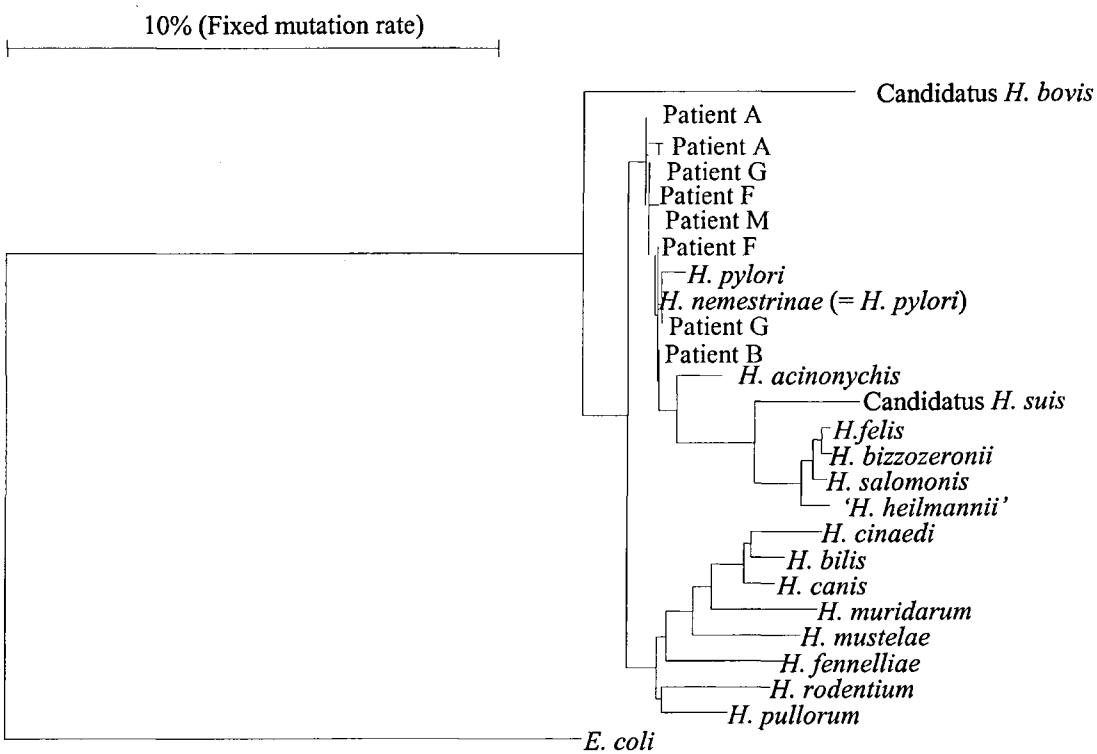



**Figure 9.2: Rooted tree illustrating the relationships between 16S rRNA of 17 *Helicobacter* species and sequences generated from bladder biopsies.**

Alignment of the 16S sequence determined from patient E, following amplification by the assay of Fox *et al* (1995), with primer pairs for the other four assays applied

demonstrated a mismatch between template and the primer Hp2 of Ho *et al* (1992). This is illustrated in Figure 9.3.


**Figure 9.3: 16S rRNA sequence amplified from the bladder of Patient E aligned with primers of the assays of Logan, Bohr and Ho.**

Alignment with primers of Ho *et al* (1992) demonstrates a mismatch between template DNA and the 3' end of sense primer Hp2*.

### 9.2.5 PCR-based analysis of colonic biopsies

All colonic biopsies were tested by the same panel of *Helicobacter* genus and species-specific PCR assays as described above for the bladder biopsies. The 16S rDNA assay of Bohr *et al* (1998) generated amplicons of approximately 639 bp in 12 biopsies. However, amplicon quantity was insufficient for sequencing, and subsequent enhanced band separation in a 3.0 % agarose gel demonstrated that the bands were slightly larger than that of the positive control. Amplicons were not generated by any of the other assays applied.

### 9.2.6 PCR-based analysis of bronchial biopsies

All bronchial biopsies were tested for helicobacters by the same panel of PCR assays. No specific amplicon was generated in any of the biopsies tested.

## 9.3 Discussion

As discussed in section 1.9, PCR has proved to be a key technique in the investigation of the role of helicobacters in extra-gastric diseases. The majority of these studies amplified a single target with one primer pair (single round PCR) (Birek *et al.* 1999;Riggio and Lennon 1999;Roe *et al.* 1999;Tanaka *et al.* 1999) or two primer pairs in a nested format (Blasi *et al.* 1996;Danesh 1998;Fanning *et al.* 1998;Kawaguchi *et al.* 1996;Lin *et al.* 1995;Mravak-Stipetic *et al.* 1998). As discussed in Chapter 3, reliance on a single PCR detection assay can lead to falsely negative results due to heterogeneity at the primer binding site, and performance of individual PCR assays can be differentially affected by factors such as specimen quality. Results presented in Chapter 8 highlighted the issue of non-specific amplification that can arise with nested PCR. Furthermore, the additional specimen manipulation of a second round of PCR increases the risk of contamination and the

occurrence of false-positive results. These problems are particularly difficult to recognise in the absence of a "gold standard" method, such as culture, which would allow validation of a PCR assay for a novel application. In this chapter, PCR assays were used to investigate chronic inflammatory conditions of the genitourinary tract (IC), the lower gastrointestinal tract (CD and UC) and the lower respiratory tract (CPC). In contrast to most studies conducted to date, a panel-based approach comprising two *Helicobacter* genus-specific and three species (*H. pylori* and HHLOs)-specific PCR assays was used. This multiple assay and target (16S rRNA and *vacA*) approach was adopted to overcome the problems of false negatives discussed above, but also so that inter-assay correlations would corroborate any PCR-positive results.

### 9.3.1 PCR-based detection of helicobacters in bladder biopsies

Clinical features such as: rapid onset of disease; higher incidence in women; a lack of family history or HLA haplotype-association and presence of IgA deposits in the bladder epithelium suggest IC may have a bacterial aetiology (Haq *et al.* 2001). Several broad-based PCR studies that universally amplified all bacterial 16S rRNA have been conducted to investigate this hypothesis, and bacteria including *E. coli* and species of *Prevotella*, *Actinomyces*, *Propioniobacterium* and *Acinetobacter* have been detected in bladder biopsies (Domingue *et al.* 1995;Heritz *et al.* 1997;Keay *et al.* 1998b). In contrast, other studies have been unable to detect any bacterial DNA at this site (Haarala *et al.* 1996;Hampson *et al.* 1993). No association between bacterial DNA and IC was demonstrated in any of these single PCR assay-based studies. The possibility that PCR assays failed to detect *Helicobacter* due to poor assay sensitivity cannot be excluded and consequently the hypothetical bacterial aetiology of IC remains unresolved.

In this chapter, multiple genus and species-specific PCR assays were applied to investigate the potential role of *Helicobacter* in IC for the first time. PCR analyses demonstrated that 85.2 % of biopsies were PCR-positive by at least one assay. However, inter-assay correlation was poor, with some biopsies positive for 4/5 assays while others were positive by only one of the two *Helicobacter*-specific assays. The poor inter-assay corroboration raised concerns that amplification was non-specific, particularly as only two of the patients (F and R) were actually seropositive for *H. pylori*. Furthermore biopsies from just two patients (B and M) were PCR-positive for the HpVac assay targeting *vacA*; a gene that is present in all *H. pylori* isolates (Cover 1996). As demonstrated in Chapter 3, the sensitivity of different PCR assays varies considerably, particularly if initial target DNA levels are low, as suggested by the low-intensity bands generated in many bladder biopsies. Testing of serial tenfold dilutions of *H. pylori* DNA (NCTC 11637 and NCTC 11916) demonstrated that the assay of Logan *et al* (2000) was ten times more sensitive than that of Bohr *et al* (1998), detecting as little as 100 fg. Such differences in assay performances could at least partially contribute to discrepancies between tests.

To facilitate interpretation of results, an additional prokaryote-specific PCR assay described by Fox *et al* (1995) was applied to all biopsies. The failure of this assay to generate prokaryotic amplicon in 11 patients that were weakly positive by the Bohr assay, suggested the latter had non-specifically amplified non-bacterial DNA, that was possibly of human origin. Thus, the Bohr assay may not be sufficiently specific for application to human tissue, a conclusion that has since been confirmed by the original author in a later study (Bohr *et al*, 2002). Furthermore, sequence data generated by the assay of Fox *et al* (1995) for patient E identified sequence variation at the Ho *et al* (1992) primer Hp2 binding site, and this may account for inter-assay

discrepancies also. This observation again demonstrates that no single PCR assay can detect all strains of a bacterial species or genus, particularly one like *Helicobacter* that is highly heterogeneous at the genome level. The potential limitations of a single PCR-assay approach were highlighted also by one other study of IC, published in June 2003, that failed to amplify *H. pylori*-specific DNA in paraffin-fixed bladder biopies from 33 IC patients using the same Ho 16S rRNA PCR assay (Agarwal and Dixon, 2003). While this may suggest that *H. pylori* is not present in the human bladder, the negative PCR results reported may be due to insufficient sensitivity of the assay applied, as was observed for the study presented in this chapter.

### 9.3.2 Characterisation of amplified products from bladder biopsies

BLASTn alignment of sequenced 16S rDNA amplicons demonstrated that these were >99 % similar to *H. pylori* while amplicons of the less-conserved *vacA* gene were ≤ 97 % similar. This confirmed that PCR amplicons were not spurious and suggested that *H. pylori* was present in the human bladder. However, it should be noted that studies that rely on PCR based results alone in combination with sequencing data may misidentify the species detected. BLASTn searches, although useful, have the limitation that they can only align novel unknown sequences with a list of sequences currently held in public databases. A vast number of commensal bacterial species are likely to colonise humans that have either not yet been identified, or characterised by sequencing. Such sequences thus could be misidentified by BLASTn analysis. For this reason it is preferable to adopt a multiple target approach to increase the probability of correct identification of sequences and thus accurate speciation. One additional advantage of the prokaryote-specific assay was that the entire 16S sequence spanning variable regions V1 to V9 (illustrated in Figure 1.3) could be determined in two patients. The chances of such a large DNA sequence being misidentified by

BLASTn analysis is extremely low and so this data provided compelling evidence of amplicon specificity.

As demonstrated in Chapter 8, *H. pylori* is often present in the stool of infected individuals, although it is not known if cells are intact or fragmented. It is hypothesised that, like most uropathogens, *H. pylori* may enter the bladder by an ascending route, although matched patient stool samples were not available to explore this possibility. Nevertheless, as several of the control patients were also PCR-positive, the association between *H. pylori* and development of IC cannot be supported. This finding is substantiated by studies that found no increased *H. pylori* seroprevalence in IC patients (English *et al.* 1998) or failed to demonstrate higher numbers of CLO test-positive results in bladders of IC patients (Haq *et al.* 2001). Culture of these biopsies in the primary laboratory (Chelmsford PHL) had been unsuccessful, thus essential corroborating evidence of *H. pylori* in the bladder was not available, so the possibility of specimen contamination with exogenous *H. pylori* DNA could not be excluded. Iatrogenic specimen contamination at cystoscopy via forceps was demonstrated previously in a PCR-based study (Keay *et al.* 1998a), and cross-contamination of gastric biopsies at endoscopy is well-documented (Akamatsu *et al.* 1996;Debongnie and Bouckaert 1993;Miyaji *et al.* 1995). However, enteric bacteria commonly associated with bladder infections would be a more likely cause of cross-contamination at cystoscopy than *H. pylori*. Alternatively, *H. pylori* DNA could be introduced during culture of biopsies at the primary laboratory, or during DNA extraction. This would seem unlikely given that over 200 *H. pylori*-negative gastric biopsies were processed under identical conditions in the course of this study without any similar problems. Furthermore, contamination at the PCR stage was unlikely also, as none of the negative controls contained spurious amplicons.

Amplification and sequencing of 16S rDNA from various clinical specimens has been key in defining the aetiology of other conditions including Whipple's disease, bacillary angiomatosis and human ehrlichiosis (Wilson 1994). However in all these examples, additional corroborating evidence of the infectious agent (e.g. culture or visualisation by microscopy) was available also. Further work to examine other methods that would supplement the PCR-based evidence presented in the current study and allow *in situ* visualisation of infecting bacteria (e.g. histology, *in situ* hybridisation or *in situ* PCR), would provide a context for interpretation of PCR results and provide more compelling evidence that *H. pylori* was present in the human bladder. Extension of this study using these additional methods to examine a broader spectrum of diseases states could provide insight into the biological significance of helicobacters at this site.

### 9.3.3 Investigation of colonic biopsies

One study demonstrated the presence of helicobacters in colonic biopsies from CD patients by a broad range prokaryote-specific 16S rDNA PCR, followed by specific probe hybridisation and sequencing approach (Tiveljung *et al.* 1999). The authors speculated that helicobacters act as ulcerative pathogens, contributing to the pathogenesis of CD, possibly as part of a mixed microbial infection. *Helicobacter* infection was confirmed by hybridisation of a specific probe spanning the hypervariable region V4 of 16S rDNA. Sequencing was unsuccessful. Assay specificity is particularly important in specimens like colonic biopsies that contain high levels of competing bacteria that may cross-react with some primer pairs. The work presented in this chapter describes, for the first time, the application of *Helicobacter* genus and species specific PCR assays in the investigation of the

aetiology of CD and UC. Multiple assays were applied to facilitate interpretation of any positive PCR results.

In contrast to the results obtained for the bladder biopsies, application of the same PCR assays to colonic biopsies did not generate product in the majority of cases. In 12/30 colonic biopsies, faint bands of approximately 639 bp were generated by the *Helicobacter* genus-specific assay of Bohr *et al*, 1998. These amplicons could not be characterised further as insufficient product was generated for sequencing. However improved resolution of these bands showed they were larger than the anticipated *Helicobacter*-specific product. This observation, combined with the fact that no other PCR assay generated amplicons, suggested that these bands were non-specific, as was observed in the bladder biopsies. Thus, colonic biopsies were PCR negative for all assays applied. The poor specificity of the Bohr PCR assay highlights the problems of interpreting positive results from a single PCR assay not previously validated for that application, particularly if insufficient product is generated to allow confirmatory testing. The multiple assay/target approach described in this chapter quickly allowed false-positive results to be excluded.

Negative results could also have occurred due to insufficient assay sensitivity. The lack of a gold standard method to allow assessment of sensitivity of these assays when applied to colonic biopsies, prevents exclusion of this possibility. Although no helicobacters were observed by histological analyses of biopsies, specimens were stained by routine haematoxylin and eosin. This method is relatively insensitive for detection of helicobacters compared with, for example, Giemsa or Warthin Starry staining (El Zimaity 2000). Further investigations might include appropriate histological analysis of biopsies and application of a nested PCR to address the sensitivity issue, but interpretation of PCR results can be difficult, particularly when

specimens are from a site rich in diverse bacterial species. Previously, a lower incidence of *H. pylori* infection in patients with IBD was demonstrated, particularly in those who had taken sulphasalazine, suggesting that IBD drug therapy may inhibit bacterial growth (Parente *et al.* 1997b). However, non sulpha 5ASA medication did not have the same inhibitory effect (el Omar *et al.* 1994). In this chapter, only one UC patient was taking sulphasalazine; while 9/11 with UC and 1/9 with CD were prescribed non sulpha 5ASA (S. Bell, personal communication). Consequently, it seems unlikely that the negative PCR results were due to bacterial inhibition or eradication by IBD specific medication. The results suggest that helicobacters do not play a significant role in CD or UC, a conclusion that is also supported by previous serological based studies that found a negative association between *H. pylori* infection and IBD (Halme *et al.* 1996;Kolho *et al.* 1998;Parente *et al.* 1997a;Pearce *et al.* 2000). Recently, a German study described a novel 16S rRNA PCR assay using modifications of primers C97 and HGR (section 1.7.1.1) that amplified fragments of DNA from intestinal biopsies of 2 of 3 CD patients, but not in any of the three UC patients examined (Bohr *et al.* 2002). Sequence analyses identified amplicons as *H. pylori* and *H. pullorum*. It is not known if these results indicate higher sensitivity of the novel PCR assay compared with those applied in this chapter or if differences in detection rates are due to geographical variation, but this could be assessed by future application of this assay to the colonic biopsies from English patients. As few biopsies were examined in the Bohr study, the significance of these PCR results in relation to IBD remains unknown.

As discussed in sections 1.5.2.3 and 1.8.2, studies, including that presented in Chapter 8, have shown that *H. pylori* is detectable in the lower intestine, by demonstration of specific antigens or DNA in stools. It is not known if *H. pylori* in

stools interact with the mucosa of the large intestine, leading to development of disease of the lower gastrointestinal tract. A previous study using nested 16S rDNA PCR to investigate the role of *H. pylori* in appendicitis failed to detect *H. pylori* in intestinal biopsies (Fanning *et al.* 1998), despite the enhanced sensitivity of this format. The results in this chapter also suggest that *H. pylori* and other helicobacters are not associated with the intestinal mucosal surface. However, further investigations of IBD patients of known *H. pylori* status will be essential to substantiate this observation.

### 9.3.4 Investigation of bronchial biopsies

PCR-based analyses of bronchial biopsies failed to detect helicobacters in any of the 10 patients investigated. Internal control PCR demonstrated that negative results were not due to inhibition of *Taq* polymerase and as multiple assays targeting different areas of the genome were applied, there is no evidence that helicobacters contributed to CPC in these patients. However, it should again be appreciated that this possibility cannot be wholly excluded, as bacterial DNA may be present below the threshold of detection of the PCR assays. Other studies have adopted a nested PCR approach to improve sensitivity of detection, this was not attempted here, given the increased risk of contamination that can be difficult to identify in samples for which there is no gold standard for comparison or assay validation. As for the bladder and colonic biopsies, future investigations should focus on application of more sensitive (nested) methods of PCR-based detection as well as confirmatory evidence provided by direct visualisation of helicobacters *in situ*.

## 9.4 Conclusions

The work presented in this chapter showed for the first time that helicobacters may be present in the human bladder, although the clinical significance of this remains to be established. In contrast, no evidence of helicobacters in colonic or bronchial biopsies was provided following multiple analyses of samples with *Helicobacter* genus and species-specific PCR assays.

One cautionary point raised by this study is that it is difficult to fully exclude the possibility of contamination with extrogenous bacteria or bacterial DNA, if evidence of an organism's presence is based on PCR alone. Further evidence of the infecting organisms, ideally by culture but also for example by histology or by *in situ* hybridisation would corroborate any PCR-positive results and provide more compelling evidence of potential associations with disease. Consideration of this issue for future investigations will greatly improve the quality of experimental outcomes.

# Chapter 10: Application of PCR in the investigation of the mechanism of metronidazole (MTZ) resistance in *H. pylori*.

## 10.1 Background

In Chapter 5, PCR proved to be a powerful, rapid means for determination of clarithromycin (CLA) susceptibility. MTZ is also a key antibiotic in *H. pylori* eradication, but the utility of this is now compromised by high resistance rates world-wide (Alarcon *et al.* 1999;Megraud *et al.* 1999). Surveillance of MTZ resistance is problematic as there are no standardised methods for culture-based susceptibility testing. Likewise, the potential development of a simple molecular test for determining MTZ susceptibility analogous to that for CLA has been hindered by poor understanding of the mechanism of MTZ action and of resistance development in *H. pylori* (Jenks and Edwards 2002;Mendz and Megraud 2002).

As discussed in section 1.11.6, current evidence suggests that genes *rdxA* and *frxA*, encoding an oxygen-insensitive NADPH nitroreductase and an NADPH flavin oxidoreductase, respectively, may be involved in MTZ metabolism and that resistance can develop by mutational inactivation of these (Debets-Ossenkopp *et al.* 1999;Kwon *et al.* 2000d;Kwon *et al.* 2000b;Kwon *et al.* 2001b;Solca *et al.* 2000). However, reports of MTZ-R strains that possess apparently unaltered *rdxA* and *frxA* compared with MTZ-S strains (Goodwin *et al.* 1998) indicates possible involvement of other genes (Kwon *et al.* 2001a;Tankovic *et al.* 2000;Wang *et al.* 2001). Two recent studies have provided protein-based evidence that a homologue of subunit AhpC of alkyl hydroperoxide reductase may be involved in MTZ resistance also (McAtee *et al.* 2001;Trend *et al.* 2001).

In this chapter, PCR-based methodologies were applied to investigate *rdxA*, *frxA* and *ahpC* in a unique collection of 50 clinical *H. pylori* isolates recovered from 21 patients in England undergoing investigation for symptoms of dyspepsia.

The aims of this study were:

1) To identify mutations in *rdxA*, *frxA* and *ahpC* in paired MTZ-S/MTZ-R isolates that may contribute to MTZ resistance in the UK.

2) To examine mixed MTZ-S/MTZ-R infections by PCR-based genotyping to evaluate the contribution of mixed genotype infections to treatment failure.

3) To compare gene sequences of MTZ-R strains recovered before and after therapy with matched pre-treatment MTZ-S strains (and MTZ-R strains, if present) to establish if treatment had failed due to selection of a pre-existing mutated MTZ-R strain or if *de novo* mutation of both the MTZ-S and MTZ-R sub-populations, leading to higher resistance levels, occurs during the eradication regime.

4) To determine by a novel real-time PCR assay the frequency of early frameshift mutations in *frxA* and to assess the relationship of these to MTZ susceptibility.

## 10.2 Results

### 10.2.1 Study population

Isolates cultured from gastric biopsies of 21 dyspeptic patients from Ipswich (A – H) and London (G – U), England were included in this study. Thirteen of these patient sets were stored paired cultures (patients A – M), recovered from patients before and after they had received MTZ-based eradication therapy (Owen *et al.* 1993). A further four were strain pairs recovered pre-treatment from single gastric biopsies of dyspeptic patients (N – Q) that were mixed MTZ susceptibility, and the remaining

four were isolate pairs recovered pre-treatment from two gastric sites (antrum and corpus) of patients R – U. MTZ susceptibilities were determined for all isolates following the protocols described in sections 2.7.2. Mixed MTZ-S/MTZ-R sub-populations were separated as described in section 2.8. AFLP types were determined for all isolates as described in section 2.21. Complete or partial fragments of genes *rdxA*, *frxA* and *ahpC* were amplified by PCR (section 2.17) in all strains, amplicons sequenced and then analysed as described (2.22).

### 10.2.2 Characterisation of strain resistotype

E-testing of *H. pylori* from patients A – M showed that pre-treatment isolates were either predominantly MTZ-S with fewer MTZ-R colonies (ranging from 30 to > 100) growing in the zone of inhibition (n = 7), or fully MTZ-S (n = 4) or MTZ-R (n = 2) (Table 10.1). All patients, except for patient D, were infected with MTZ-R strains only post-therapy.

Mixed MTZ-S/MTZ-R resistotype pre-treatment infections were identified in gastric antral biopsies of patients N – Q, while pre-treatment strains with different MTZ resistotypes were found in the antrum and in the body of the gastric mucosa of patients R – U (Table 10.1). No post-treatment isolates were available for patients N – U.

### 10.2.3 Characterisation of strain genotype

AFLP analysis demonstrated that all 21 patients were infected by *H. pylori* with unique genotypes (Table 10.1). Examples of the AFLP profiles generated are shown in Figure 10.1. AFLP genotypes determined for isolate pairs from the 12 patients (A, B, D, E, H, I, K, M – Q) with mixed (MTZ-S/MTZ-R) infections recovered from a single gastric site (pre-treatment, except for patient D) were either identical (n = 10) or similar, differing by a single band (n = 2), and isolate pairs of mixed (MTZ-

S/MTZ-R) infection recovered from patients R – U (pre-treatment) from two separate gastric sites (antrum and corpus) had identical AFLP genotypes. Comparison of AFLP genotypes of pre-treatment isolates with those recovered post-treatment in patients A – M demonstrated that these were mainly identical (n = 8) or similar (n = 3), while distinct genotypes, differing by two or more bands, were identified post-treatment in patients H and I (Table 10.1).

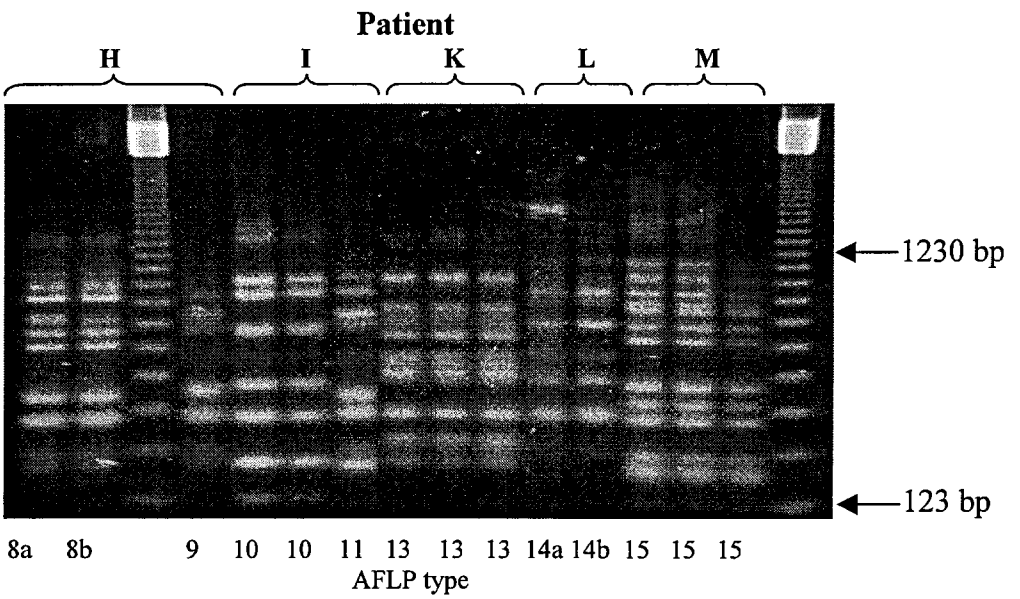



**Figure 10.1: Examples of different AFLP profiles generated for paired MTZ-S and MTZ-R isolates.** For all patients, AFLP types are in the order MTZ-S, MTZ-R (pre-treatment) and MTZ-R (post treatment), except where mixed susceptibility strains were not observed pre-treatment (patient L). 123 bp molecular weight marker (lanes 3 &16).

**Table 10.1: MTZ resistotypes and AFLP genotypes of 50 strains from 21 patients**

| Gastric Site | Patient Code | Stage of treatment | MTZ MIC (µg/ml) | MTZ Susceptibility | AFLP Profile* |
|---|---|---|---|---|---|
| Antrum | A | Pre | 0.25 + >256 | S + R | 1 , 1 |
| | | Post | >256 | R | 1 |
| | B | Pre | 0.19 + 16 | S + R | 2a , 2a |
| | | Post | >256 | R | 2b |
| | C | Pre | 0.094 | S | 3 |
| | | Post | >256 | R | 3 |
| | D | Pre | 0.19 | S | 4a |
| | | Post | 0.25 + 64 | S + R | 4b , 4c |
| | E | Pre | 0.25 + >256 | S + R | 5 , 5 |
| | | Post | >256 | R | 5 |
| | F | Pre | 0.064 | S | 6 |
| | | Post | >256 | R | 6 |
| | G | Pre | 96 | R | 7 |
| | | Post | >256 | R | 7 |
| | H | Pre | 0.5 + 128 | S + R | 8a + 8b |
| | | Post | 48 | R | 9 |
| | I | Pre | 0.25 + >256 | S + R | 10, 10 |
| | | Post | >256 | R | 11 |
| | J | Pre | 96 | R | 12 |
| | | Post | >256 | R | 12 |
| | K | Pre | 0.25 + >256 | S + R | 13 , 13 |
| | | Post | >256 | R | 13 |
| | L | Pre | 0.38 | S | 14a |
| | | Post | 16 | R | 14b |
| | M | Pre | 0.5 + 256 | S + R | 15 , 15 |
| | | Post | >256 | R | 15 |
| | N | Pre | 0.19 + >256 | S + R | 16 , 16 |
| | O | Pre | 0.125 + >256 | S + R | 17 , 17 |
| | P | Pre | 0.125 + >256 | S + R | 18 , 18 |
| | Q | Pre | 0.25 + >256 | S + R | 19 , 19 |
| Antrum and Corpus | R | Pre | 0.25 + 48 | S + R | 20 , 20 |
| | S | Pre | 0.094 + 12 | S + R | 21 , 21 |
| | T | Pre | 0.064 + >256 | S + R | 22 , 22 |
| | U | Pre | 2 + >256 | S + R | 23 , 23 |

*Unrelated AFLP profiles are identified by different (arbitrary) numbers; similar but non-identical fingerprints are distinguished by lower-case letters (a, b, and c).

### 10.2.4 Comparison of translated RdxA sequences in MTZ-S and MTZ-R strains

Comparison of all isolates with sequences held in GenBank (Appendix C.1) identified novel mutations, as well as those described previously (Solca *et al.* 2000) (Table 10.2).

#### 10.2.4.1 Comparison of matched pre and post treatment RdxA sequences

Of the nine matched pre and post-therapy MTZ-S and MTZ-R strains of similar AFLP genotypes (A – F, K – M), the inferred RdxA amino acid sequences were different in seven patients (A – C, E, F, L, M) (Table 10.2). Nucleotide point mutations (corresponding to a substitution of Arginine-16 to Histidine in three of five cases) occurred in MTZ-R strains from patients A, B, E, F and L. Frameshifts leading to protein truncation were seen in MTZ-R isolates from patients C and M. No mutations were found in the remaining two matched (MTZ-S and MTZ-R) pairs (patients D and K). Additionally, RdxA was identical in one of the matched pre and post-treatment MTZ-R pairs (patient G), while a single amino acid substitution was found in the other pair (patient J) (Table 10.2).

#### 10.2.4.2 Comparison of matched pre-treatment MTZ-S and MTZ-R RdxA sequences

RdxA was identical in 11/16 MTZ-S and MTZ-R sub-population sets, recovered at the pre-treatment stage in all but one case (patient D), either from a single or from two gastric sites (n = 12 and 4, respectively). Mutations in the MTZ-R sub-population only (patients H, I, N and O) included stop codons (n = 3) and carboxy terminus sequence alteration (downstream of residue 192) (n = 1) (Table 10.2). RdxA was truncated in both MTZ-S and MTZ-R subpopulations of patient Q (Table 10.2).

**Table 10.2: Sequence variations in *rdxA* identified by comparison of matched MTZ-S and MTZ-R strains of *H. pylori* recovered from patients before and after therapy or simultaneously as a mixed infection.**

| *rdxA* mutation type (nucleotide position) | | | RdxA amino acid sequence change (codon) | Patient |
|---|---|---|---|---|
| Pre-treatment | | Post-treatment | | |
| MTZ-S | MTZ-R | MTZ-R | | |
| No mutation | No mutation | **Substitution* (G47A)** | **Arg→His (16)** | A, B, E |
| No mutation | No mutation | No mutation | None | K |
| No mutation | | No mutation/† No mutation | None | D |
| No mutation | No mutation | Frameshift (+T328) | Stop codon (110) | M |
| No mutation | Substitution (G319T) | ND‡ | Stop codon (107) | H |
| No mutation | **Substitution (C148T)** | ND‡ | **Stop codon (50)** | I |
| No mutation | NA§ | **Frameshift (+A197)** | **Stop codon (74)** | C |
| No mutation | NA§ | Substitution (C146G) | Thr→Arg (49) | F |
| No mutation | NA§ | Substitution (C200T) | Ala→Val (67) | L |
| NA§ | No mutation | No mutation | None | G |
| NA§ | No mutation | Substitution (C131T) | Pro→Leu (44) | J |
| No mutation | No mutation | NA | None | P, R – U |
| No mutation | Frameshift (+T575) | NA | (193 onwards) | O |
| No mutation | Frameshift (+AT151) | NA | Stop codon (56) | N |
| **Substitution (C148T)** | **Substitution (C148T)** | NA | **Stop codon (50)** | Q |

*Mutations in bold font have been reported previously (Solca *et al.* 2000).

†A mixed MTZ-S/MTZ-R infection was observed post-treatment in patient D, where *rdxA* sequence was identical in all strain variants.

‡Comparison not done as post-treatment strains were different AFLP types.

§Not applicable as no mixed MTZ-S/MTZ-R infections were identified.

### 10.2.5 Comparison of translated AhpC sequences in MTZ-S and MTZ-R strains.

Interstrain variation of as much as 5 % was observed in AhpC amino acid sequences but no sequence differences were found in any of the populations of each patient strain set. Examples of matched patient set AhpC amino acid sequences are presented in Figure 10.2.

```
Patient A1   1   MLVTKLA DF KA AVLGNNE VDE FELSKN LGKSGAILFF W KDFTFVC  TEIIAFDKRV  60
Patient A2   1   ------- -- -- ------- --------- ---------- - -------  ----------  60
Patient A3   1   ------- -- -- ------- --------- ---------- - -------  ----------  60
Patient B1   1   ----T-- -- -- ------- --------- ---------- - -------  ----------  60
Patient B2   1   ----T-- -- -- ------- --------- ---------- - -------  ----------  60
Patient B3   1   ----T-- -- -- ------- --------- ---------- - -------  ----------  60
Patient E1   1   ------- -- -- ------- --------- ---N------ - -------  ----------  60
Patient E2   1   ------- -- -- ------- --------- ---N------ - -------  ----------  60
Patient E3   1   ------- -- -- ------- --------- ---N------ - -------  ----------  60

Patient A1  61   KDFQEKGFNV IGVSIDSEQV FAWKNT VE KGGIGQVTF  MVADITKSIS RDYDVLFEEA  120
Patient A2  61   ---------- ----------  ------ -- ---------  ---------- ----------  120
Patient A3  61   ---------- ----------  ------ -- ---------  ---------- ----------  120
Patient B1  61   ---------- ----------  ------ -- ---------  ---------- ----------  120
Patient B2  61   ---------- ----------  ------ -- ---------  ---------- ----------  120
Patient B3  61   ---------- ----------  ------ -- ---------  ---------- ----------  120
Patient E1  61   ---------- ----------  ------ -- ---------  ---------- ----------  120
Patient E2  61   ---------- ----------  ------ -- ---------  ---------- ----------  120
Patient E3  61   ---------- ----------  ------ -- ---------  ---------- ----------  120

Patient A1 121   IALRGAFLID KNMKVR AVI NDL LGRNAD EMLRMVDALL FEE GEVC  AGWRKGDKGM  180
Patient A2 121   ---------- ------ --- --- ------ ----------  ---- ----  ----------  180
Patient A3 121   ---------- ------ --- --- ------ ----------  ---- ----  ----------  180
Patient B1 121   ---------- ------ --- --- ------ ----------  ---- ----  ----------  180
Patient B2 121   ---------- ------ --- --- ------ ----------  ---- ----  ----------  180
Patient B3 121   ---------- ------ --- --- ------ ----------  ---- ----  ----------  180
Patient E1 121   ---------- ------ --- --- ------ ----------  ---- ----  ----------  180
Patient E2 121   ---------- ------ --- --- ------ ----------  ---- ----  ----------  180
Patient E3 121   ---------- ------ --- --- ------ ----------  ---- ----  ----------  180

Patient A1 181 KA 182
Patient A2 181 -- 182
Patient A3 181 -- 183
Patient B1 181 -- 182
Patient B2 181 -- 183
Patient B3 181 -- 183
Patient E1 181 -- 183
Patient E2 181 -- 183
Patient E3 181 -- 183
```

***Strains numbered: 1 = MTZ-S (pre-therapy); 2 = MTZ-R (pre-therapy); 3= MTZ-R (post-therapy)**

**Figure 10.2: Examples of partial AhpC amino acid sequences in matched patient strain sets of separated MTZ-S and MTZ-R sub-populations (pre-treatment) and MTZ-R populations (post-treatment)**

### 10.2.6 Comparison of translated FrxA sequences in MTZ-S and MTZ-R strains.

As discussed earlier (section 2.17.2), various primer combinations were required to amplify *frxA* for sequencing. This allowed determination of the complete *frxA* gene in 20/21 patients, but amplification was unsuccessful for patient R.

In 16/20 (80.0 %) patient sets examined, no mutational differences were observed in any of the matched populations, while frameshift mutations were observed only in MTZ-R populations in three patients (C, K, L) and in the MTZ-S strain only in patient T. Multiple alignment of FrxA sequences from this study with 23 sequences held in GenBank (Appendix C.2) showed that at least one strain from 18 of the 20 (90.0 %) patient sets had a frameshift mutation that led to premature truncation of FrxA protein (Table 10.3, Figure 10.3). In 14/18 patient sets, frameshift mutations were observed in all strains, regardless of MTZ resistotype. Frameshifts occurred at nucleotide 53 in 12/18 (66.7 %) patient sets, usually due to a single adenine deletion. In most cases this was predicted to lead to early protein truncation at codon 39 (Figure 10.3), with the exception of patient P where a G117T substitution altered codon 39 and also patient N where a 2-bp (AA) deletion was observed at position 53 (Figure 10.3).

```
Patient F  1   MDREQVVALQ HQRFAAKKYD SNRRISQKDW EALVEVGRLA  SSIGLE WK MLLLKNERMK  60
Patient B  1   MDREQVIALQ HQRFAAKKYD  NRRISQKDW EALVEVGRLA  SSIGLE WK MLLLKNERMK  60
Patient M  1   MDREQVVALQ HQRFAAKKYD  NRRISQKDW EALVEVGRLA  SSIGLE WK MLLLKNERMK  60
Patient C  1   MDREQIIALQ HQRFATKKYD  NRRISEKDW EVLVEVGRLA  SSIGLE WK MLLLKNERMK  60
                                  Δ-A*
Patient P  1   MDREQVVALQ HQRFAAKNTI LIVVF KRIG KLWLKWGDY  LLQSGLNHGK CFY-        53
                                 Δ-A*
Patient A  1   MDREQVVALQ HQRFAAKNTI LIVVF KRIG KLWLKWGD-                         38
                                 Δ-AA*
Patient N  1   MDREQVVALQ HQRFAAKIRS -                                           20

Patient F  61  EDLK MAWGA LFGLEGASHF VIYLARKGVT YDSDYVKKVM HEVKKRDYDT NSRFAQIIKN  120
                      Δ-T*
Patient B  61  EDLK MAWGA LFWFGGSE F C LSCAKRRY L-                               91
                     Δ-CT*
Patient M  61  EDLK MAWGA FWFRGSE FC YLSCAKRRYL -                                 90
                   Δ-G*
Patient C  61  EDLK MAWGG FLV-                                                   73

Patient F  121 FQENDMKLNS ERSLFDWASK QTYIQMANMM MAAAMLGIDS C IEGYDQEK VEAYLEEKGY  180

Patient F  181 LNTAEFGVSV MACFGYRNQE IT KTRWKTE VIYEVIE- 217
```

**Figure 10. 3: Examples of FrxA amino acid sequences determined for each frameshift mutation type observed in different patient sets**

*Nucleic acid sequence changes that caused amino acid alterations e.g. for patient P, one adenine deletion in the nucleotide sequence caused frameshift leading to an altered amino acid sequence and creation of a stop codon.

**Table 10.3: Sequence variations in *frxA* identified by comparison of matched MTZ-S and MTZ-R strains of *H. pylori* recovered from patients before and after therapy or simultaneously as a mixed infection**

| *frxA* mutation (nucleotide position) | | | FrxA amino acid sequence change (codon) | Patient |
|---|---|---|---|---|
| Pre-treatment | | Post-treatment | | |
| MTZ-S | MTZ-R | MTZ-R | | |
| Frameshift (53) | Frameshift (53) | Frameshift (53) | Stop codon (39) | A, D, E |
| Frameshift (53) | Frameshift (53) | NA* | Stop codon (39) | H, I |
| Frameshift (212) | Frameshift (212) | Frameshift (212) | Stop codon (92) | B |
| Frameshift (211) | Frameshift (211) | Frameshift (211) | Stop codon (91) | M |
| No mutation | Frameshift (209) | Frameshift (209) | Stop codon (74) | K |
| No mutation | NA† | Frameshift (209) | Stop codon (74) | C |
| No mutation | NA† | Frameshift (53) | Stop codon (39) | L |
| Frameshift (53) | Frameshift (53) | NA‡ | Stop codon (21) | N |
| NA§ | Frameshift (53) | Frameshift (53) | Stop codon (39) | G |
| No mutation | NA† | No mutation | None | F |
| NA§ | No mutation | No mutation | None | J |
| Frameshift (53) | Frameshift (53) | NA‡ | Stop codon (39) | Q, S, U |
| Frameshift (24) | Frameshift (24) | NA‡ | Stop codon (39) | O |
| Frameshift (53) | Frameshift (53) | NA‡ | Stop codon (54) | P |
| Frameshift (24) | No mutation | NA‡ | Stop codon (39) | T |

*Not applicable as post-therapy strains had been shown to be a different AFLP genotype.

†Not applicable as mixed susceptibility infections were not observed.

‡Not applicable as no post-treatment isolates were available.

§Not applicable as only MTZ-R strains were observed pre-treatment.

### 10.2.7 Mutations in *rdxA* and *frxA* in relation to MIC

The combined incidences of *rdxA* and *frxA* mutations for each patient set is summarised in Table 10.4. This revealed that 8/21 (38.1 %) patients (A, B, E, H, I, M

- O) infected with highly resistant isolates (MIC >128 μg/ml) had mutations in *rdxA* of MTZ-R strains (either both pre and post-treatment or after therapy only) and in *frxA* of all populations. Two patient sets (C and L) had mutations in *rdxA* and *frxA* of MTZ-R isolates only, but the MIC of the MTZ-R isolate from one of these was comparatively low (16 μg/ml). Two other patient sets (F and J) had mutations in the *rdxA* only of the MTZ-R subpopulations that had high MICs (>256 μg/ml). A further 33.3 % (7/21) of patient isolates (D, G, K, P, S – U) had mutated *frxA* genes only, although 5/7 were highly resistant (MIC >256 μg/ml).

**Table 10.4 The effects of combined *rdxA* and *frxA* mutations in relation to MIC**

| ***rdxA* mutations** | | | ***frxA* mutations** | | | **Patient** | **Range of MICs for MTZ-R strains** |
|---|---|---|---|---|---|---|---|
| **S+R** | **S only** | **R only*** | **S+R** | **S only** | **R only*** | | |
| - | - | ✓ | ✓ | - | - | A,B,E,H,I,M,N,O | >128 |
| - | - | ✓ | - | - | ✓ | C,L | 16 - 256 |
| - | - | ✓ | - | - | - | F,J | >256 |
| ✓ | - | - | ✓ | - | - | Q | >256 |
| - | - | - | - | - | ✓ | K,G | >256 |
| - | - | - | ✓ | - | - | D,P,S,U | 12 - 256 |
| - | - | - | ND† | ND | ND | R | >256 |
| - | - | - | - | ✓ | - | T | >256 |

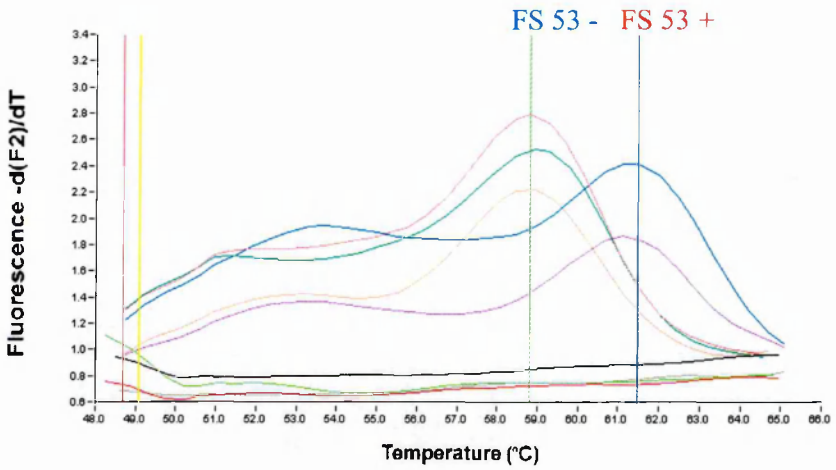
*Mutations in MTZ-R strains isolated either pre or post-treatment, or at both stages.

†Not done as *frxA* sequencing failed.

### 10.2.8 Distribution of the *frxA* frameshift 53 mutation in 119 *H. pylori* isolates

A novel LightCycler assay was developed for rapid identification of strains containing a frameshift mutation due to a single adenine deletion at nucleotide 53 as results in section 10.2.6 suggested that this was frequently observed in *H. pylori*. Probe FS-53Pr was designed to complement sequences containing this single adenine deletion (Figure 10.3). Optimisation experiments showed that probe and $MgCl_2$ concentrations of 5 pmol and 6 mM, respectively, were required to ensure sufficiently high fluorescence of probe label LC-Red 640, allowing reproducible generation of melting peaks that allowed mutant and wild-type sequences to be easily distinguished. All subsequent analyses were performed as described in section 2.20.7.

Initial evaluation of the novel LightCycler assay FS-53 on isolates (from patients A – Q and S – U) where *frxA* sequences had been determined demonstrated that sequences with a single adenine deletion at nucleotide 53, containing a run of six adenine residues, were exactly complementary to probe FS-53Pr and so generated a melting peak indicating a probe-template dissociation temperature of approximately 61 °C (Figure 10.4). Wild-type strain sequences, that retained seven adenines, were mismatched with the probe and generated a melting curve indicative of a lower dissociation temperature of approximately 59 °C (Figure 10.4). The probe failed to hybridise with sequences where the first of the seven adenine residues had been replaced with a guanine, and additional sequence variation was found (Figure 10.4).




```
FS-53Pr     1   LCRed640-ATTTGCTGCAAAAAATACGATC-P          22
Patient A   1   AGCACCAACG.....................CTAATCGTCG  42
Patient B   1   AGCACCAACG.............ATACGATCCTAATCGTCG  43
Patient C   1   AACACCAACG...C...ACG.....ATACGATCCGAATCGCCG 43

... = Sequence homology
```

**Figure 10.4: Melting peaks generated by LightCycler assay FS53 to screen for frameshift mutations at nucleotide 53, and alignment of FS-53Pr with *frxA* sequences.**

Assay FS-53 was applied to 119 miscellaneous isolates recovered pre-treatment from dyspeptic patients from London (n = 81), Wales (n = 26) mid-Essex, (n = 3), Leeds (n = 7) and Portsmouth (n = 2), that were previously characterised by E-test as MTZ-S (n = 61) or MTZ-R (n = 58). Melting peaks indicative of frameshift 53 were generated in 24/119 (20.2 %) of strains. Of the 24 strains containing frameshift 53, 14/24 (58.3 %) were MTZ-R while 10/24 (41.7 %) were MTZ-S. Distribution of frameshift mutation 53 in MTZ-S and MTZ-R strains from London, Wales and elsewhere in England is presented in Figure 10.5.

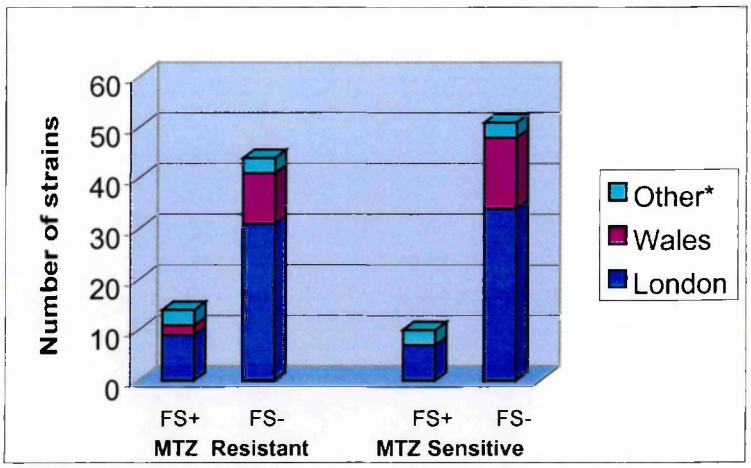



**Figure 10.5: Distribution of *frxA* frameshift mutation 53 in MTZ-R and MTZ-S strains from London, Wales and elsewhere in the UK.**

**H. pylori* from mid-Essex, Leeds, and Portsmouth (England).

## 10.3 Discussion

In this study, PCR was applied to examine the potential contribution of *rdxA, ahpC* and *frxA* to MTZ activity and resistance development in paired isolates of *H. pylori*, recovered before and after eradication therapy. Additionally, to further elucidate the significance of the observed mutations, these genes were examined pre-treatment in mixed susceptibility populations, to establish if mutations seen post-treatment were pre-existing mutations or *de novo* mutations that arose during therapy. Because this was the first investigation of isolates from the UK, the study population was extended to include paired MTZ-S and MTZ-R strains from a further eight patients, either from two gastric sites (antrum and corpus) or as part of a mixed susceptibility population from the gastric antrum. The study population included seven patient sets comprising matched pre-treatment MTZ-S and MTZ-R strains, together with matched post-treatment MTZ-R strains. This formed a unique strain set that could provide greater

insight into the potential role of *rdxA* and *frxA* in MTZ metabolism and resistance than has been possible to date. Finally, a real-time PCR assay was developed for the LightCycler to allow rapid screening of clinical isolates for single adenine deletions at nucleotide 53 in *frxA*, to assess the prevalence and significance of this mutation.

### 10.3.1 Characterisation of study population by AFLP genotyping

Evidence from studies in different countries suggests that many patients are co-infected with *H. pylori* of mixed MTZ-S and MTZ-R phenotype, but these are usually closely related strains genotypically (Dore *et al.* 1998;Jorgensen *et al.* 1996;van der Wouden *et al.* 1999). Similarly, in patient sets A – M, AFLP typing demonstrated that mixed MTZ-S and MTZ-R isolates from individual patients were phenotypic variations of the same strain rather than genotypically distinct co-infections. Isolates recovered post-therapy were in most (11/13) cases similar or identical to pre-therapy strains. Re-infection with a novel strain was infrequent, observed in two patients only. Many patients were initially infected with a MTZ-R sub-population in addition to a predominant MTZ-S population, suggesting that treatment failure occurred due to selection of the pre-existing MTZ-R population during therapy, rather than *de novo* development of MTZ resistance. Likewise, mixed MTZ resistotype infections identified in patients N – U before therapy were genotypically identical by AFLP, once more indicating that presence of a pre-existing MTZ-R strain variant was more frequently observed than mixed genotype infections.

A recent study investigating the mutagenic effects of MTZ metabolites suggested that MTZ-R colonies observed in the E-test zone of inhibition arose by spontaneous mutation and were not indicative of mixed susceptibility infections (Sisson *et al.* 2000). Furthermore, MTZ-R colonies were not observed in an agar dilution-based method that applied a serially diluted range of lower inocula (Jeong *et al.* 2000;Jeong

*et al.* 2001;Sisson *et al.* 2000). As proposed, one possible interpretation of these results is that the high bacterial cell densities used in E-testing for *H. pylori* increases the likelihood of spontaneous mutation to form MTZ-R colonies in MTZ-S populations (Jeong *et al.* 2001). However, it is equally possible that methodologies using higher-level inocula simply increase the chance of isolating a pre-existing low-level sub-population. A recent review that compared three studies (Jeong *et al.* 2001;Sisson *et al.* 2000;Wang *et al.* 2001) reported a lack of correlation between mutation rate and inoculum size (Mendz and Megraud 2002). Furthermore, routine MTZ susceptibility testing of over 400 isolates of *H. pylori* that were defined as MTZ-S in the HRU by E-test has demonstrated that the majority (> 91 %) show no evidence of MTZ-R colonies in the zone of inhibition, despite the heavy inoculum applied (R. J. Owen, personal communication). This suggests that generation of MTZ-R colonies by spontaneous mutation occurs relatively infrequently in isolates from UK patients. In contrast, MTZ-R colonies were observed in approximately 54 % of the MTZ-S isolates from patients (A – M) who had failed treatment. While these strains may be hypermutable, leading to *in vitro* induction of MTZ-R colonies, it seems more likely, given the failure of eradication therapy, that these patients were infected with a pre-existing MTZ-R sub-population. Strains isolated from the gastric antrum and the corpus in patients R – U were unequivocal examples of mixed susceptibility infections.

### 10.3.2 The role of *rdxA* inactivation in MTZ resistance

Mutations were demonstrated in 77.8 % of translated *rdxA* sequences from MTZ-R post-treatment isolates, compared with matched MTZ-S pre-treatment populations. The absence of a single universal mutation associated with MTZ resistance in UK isolates supports findings reported from other countries (Goodwin *et al.* 1998;Jenks *et*

*al.* 1999;Kwon *et al.* 2001a;Solca *et al.* 2000;Tankovic *et al.* 2000). Mutations causing Arg(16)His substitutions and protein truncations (positions 50 and 74), reported previously (Solca *et al.* 2000) could be critical to the MTZ-R phenotype (Marais *et al.* 2002). Premature protein truncation in particular would significantly reduce RdxA enzyme activity. However, these stop-codon mutations were not observed in the pre-treatment MTZ-R strain and so cannot be essential for resistance. Furthermore, no differences were observed in *rdxA* sequences of five additional paired MTZ-S/MTZ-R strains recovered from patients P and R – U before therapy. Although MTZ-R strains with unaltered *rdxA* are documented (Goodwin *et al.* 1998), this is the first report to demonstrate mutations in *rdxA* of MTZ-R strains post-therapy that are absent in matched MTZ-R and MTZ-S strains pre-therapy. Thus, MTZ-R strains with unaltered *rdxA* occur more frequently than had been hitherto indicated.

Evidence suggests that MTZ is potentially highly mutagenic (Sisson *et al.* 2000) and it may be that mutations observed in post-treatment MTZ-R *H. pylori* strains are induced during therapy, occurring coincidentally, rather than contributing to the MTZ-R phenotype. While mutationally inactivated *rdxA* genes in MTZ-R sub-populations are reported, the majority of these had been induced from progenitor MTZ-S strains by serial passage on MTZ-containing media *in vitro* (Adamsson *et al.* 2000;Jorgensen *et al.* 2001). Increasing MTZ concentrations may have induced the *rdxA* mutations reported. In contrast the MTZ-R sub-populations in this study were not induced, being observed on primary E-test.

One other study of naturally occurring mixed MTZ-S and MTZ-R populations of French and North African isolates, presumably collected pre-treatment, reported mutational differences in *rdxA* between resistotypes (Tankovic *et al.* 2000). In contrast, *rdxA* sequences were identical in most (68.8 %) mixed MTZ susceptibility

populations investigated in this study of English isolates, again suggesting that mutational inactivation of this gene is not necessary for a MTZ-R phenotype. It is difficult to account for the differences between these findings and those reported earlier (Tankovic *et al.* 2000), particularly as relatively small numbers were investigated in both studies. Differences may reflect geographic variations or even local differences in MTZ useage and *rdxA* mutation rates.

Previous transformation-based studies have provided compelling evidence to suggest an important role for *rdxA* in MTZ metabolism and in development of resistance (Goodwin *et al.* 1998;Jeong *et al.* 2000;Kwon *et al.* 2000b;Paul *et al.* 2001), as have studies where *rdxA* function has been altered by knock-out mutagenesis (Kwon *et al.* 2000a;Kwon *et al.* 2000b;Kwon *et al.* 2001b). Additionally, expression of RdxA protein is lower in MTZ-R strains (Kwon *et al.* 2000c;Latham *et al.* 2001), so a potential role for *rdxA* in MTZ resistance cannot be dismissed. However it is proposed that altered RdxA expression in MTZ-R strains may not necessarily result from functional inactivation of the gene by mutation. Control of RdxA expression may occur by an alternative regulatory mechanism, possibly at the transcription or translational level. Future investigation of this possibility in the patient strain sets described here could improve understanding of the role of *rdxA* in MTZ resistance.

### 10.3.3 The role of *ahpC* inactivation in MTZ resistance

Two recent reports provided conflicting evidence about the involvement of alkylhydroperoxide reductase (AhpC) in the MTZ resistance mechanism (McAtee *et al.* 2001;Trend *et al.* 2001). The reported differential expression of AhpC may occur as a stress response to increased toxic molecules as a result of MTZ therapy. The study presented in this chapter was the first to examine *ahpC* sequences in matched

MTZ-S and MTZ-R strains for any genotypic polymorphisms that could contribute to the MTZ resistotype. One other study failed to create an *ahpC* deficient mutant, suggesting that gene inactivation may be lethal in *H. pylori* (Lundstrom and Bolin 2000). However, it is not known if *ahpC* mutations could lead to functional modification, or to enhanced expression, of this gene. Determination of approximately 90 % of the *ahpC* gene sequence revealed that there were no differences in matched strain sequences, regardless of MTZ phenotype. Regulation of *ahpC* expression by mutational modification is therefore unlikely, and *rdxA* mutations were not linked to *ahpC* sequence alterations. However, it is possible that mutations may exist in the final 10 % of the gene. Completion of this data may prove difficult as there is limited information available about the flanking sequences of *ahpC* in *H. pylori*, that would allow primer design to amplify the entire gene. Future study of *ahpC* should address this limitation, in addition to examining *ahpC* expression at the transcription and translational level to elucidate any potential role in *H. pylori,* either alone or combined with mutated *rdxA* in MTZ resistance development.

### 10.3.4 The role of *frxA* in MTZ resistance

Translated *frxA* sequences in the same unique strain set as described above for *rdxA* were identical in most (80.0 %) of the matched patient sets. However, multiple alignment of sequences determined in this study with *frxA* sequences from non-UK isolates held in GenBank showed that frameshift mutations were common in UK isolates, whether isolated before or after therapy and regardless of MTZ resistotype. These were predicted to lead to protein truncation in all cases. Frameshift mutations in *frxA* have been reported in previous investigations that examined two MTZ-S and four MTZ-R strains (Kwon *et al.* 2000a), and a total of 21 paired isolates (Kwon *et al.* 2001a;Marais *et al.* 2003), but these were observed in MTZ-R strains only. In

contrast, results presented in this chapter demonstrate that these mutations occur in MTZ-S strains also. The differences between the results of this study and those reported previously may be attributable to either geographical variations in *frxA* sequence, or to the examination of a larger population allowing a more representative characterisation of this gene. The presence of frameshift mutations in MTZ-S strains could be interpreted as an artefact caused by MTZ-R colonies persisting after purification of MTZ-S strains by replicate and selective plating. However, this possibility was excluded by repeat sequencing of *frxA* from four MTZ-S strains, separated from MTZ-R strains by single colony expansion, which generated identical sequences to those of the original purified MTZ-S sub-populations. Additionally, repeat E-testing of all separated MTZ-S sub-populations confirmed that these were a uniform phenotype.

The results presented in this chapter suggest that inactivation of *frxA* alone does not inevitably result in MTZ resistance. This is consistent with a previous study that demonstrated that inactivated *frxA* genes did not always transform *H. pylori* phenotype from MTZ-S to MTZ-R (Jeong *et al.* 2000). Furthermore, purified recombinant FrxA protein did not reduce the MTZ drug even though *E. coli* could be transformed with *frxA* to become more sensitive to MTZ, providing evidence that FrxA does not naturally play a role in MTZ action and in resistance development (Sisson *et al.* 2002).

In the UK isolates investigated here, frameshift of *frxA* due to single adenine deletion at nucleotide 53 was the most frequently observed mutation. As the number of patient isolate sets investigated by sequencing was comparatively small and from one geographical location, a novel PCR-based probe hybridisation melting point analysis assay (FS-53) was developed for the LightCycler to allow rapid screening of

a larger, more representative, sample of MTZ-S and MTZ-R *H. pylori* isolates. Assay FS-53 allowed easy, accurate and rapid identification of strains containing the deletion mutation. Application of this assay to 119 isolates of *H. pylori* demonstrated that the prevalence of adenine deletion 53 was relatively high (20.2 %) in UK isolates. Furthermore, this mutation was observed almost as frequently in MTZ-S as in MTZ-R strains (41.7 % *vs* 58.3 %), providing further evidence that FrxA inactivation alone is unlikely to cause MTZ resistance. Results suggested that this mutation may be more common in isolates from patients in South East England, particularly in London where, compared with rural areas such as Wales, the patient population was more ethnically diverse and may have experienced increased exposure to MTZ for treatment of other infections. However, this may be a selection bias as fewer Welsh isolates were included in this investigation, so the significance of this distribution cannot be assessed at present.

Previous transformation-based studies and construction of knockout mutants have suggested that *frxA* inactivation can lead to resistance development (Jeong *et al.* 2000;Jeong *et al.* 2001;Kwon *et al.* 2000a;Kwon *et al.* 2000b;Kwon *et al.* 2001b). However, as the results presented in this chapter suggest that inactivation of *frxA* leading to protein truncation occurs frequently and does not necessarily lead to Mtz resistance, FrxA may be a non-essential enzyme. It is recognised that MTZ metabolism and resistance development in *H. pylori* is likely to be complex and multifactorial and thus the effects of an inactivated *frxA* could be compensated for by enhanced or decreased expression of other, as yet unknown, genes that have similar functions, resulting in a MTZ-S phenotype. In transformation experiments, mutated exogenous *frxA* is inserted into a naïve strain that may have no such compensatory mechanisms in place and this could result in development of phenotypic MTZ

resistance, that possibly would not occur naturally. It is evident that it is difficult to gain insight into the role of a single gene in MTZ resistance, studied in isolation, without considering the complex interplay that may exist between several genes during *in vitro* culture, and the functions these genes may have in the natural host gastric environment.

A recent study suggested that *frxA* expression may be negatively regulated by FdxA ferredoxin (Mukhopadhyay *et al.* 2003). The single adenine deletions in a polyA tract frequently observed at nucleotide 53 may indicate an additional regulatory mechanism whereby *frxA* could be switched on and off. Such homopolymeric repeats have been identified in the sequenced strain 26695 (Tomb *et al.* 1997;Saunders *et al.* 1998). Slipped strand mispairing is an important means of translational phase variation in a range of *H. pylori* genes including those involved in lipopolysaccharide synthesis (Appelmelk *et al.* 1999), the porin gene *hopZ* (Peck *et al.* 1999) and *fliP*, a gene encoding the flagellar basal body (Josenhans *et al.* 2000). The role of flavin oxidoreductase *in vivo* remains to be established, so the significance of this potential switch mechanism is unclear.

### 10.3.5 Mutations in *rdxA* and *frxA* in relation to MIC

Previous reports have suggested that mutated *frxA* may contribute to high level resistance only if combined with mutated *rdxA* (Jeong *et al.* 2000;Jeong *et al.* 2001). MICs were determined for all isolates in this study. Selective plating on CBAMTZ to purify MTZ-R sub-populations may have artificially raised strain MICs, so it is difficult to ascribe any association between *rdxA* and *frxA* mutations and MIC. Raised MICs were observed in MTZ-R populations recovered post-treatment on standard CBA from patients B, G and J. In two patients (B and J), the possibility that this may be due to the novel substitution mutations observed in *rdxA* cannot be

excluded. However, no differences were observed in *rdxA* of the MTZ-R isolate pair of patient G, in spite of the apparent increased resistance, although *frxA* mutations were identified. Mutations in MTZ-R isolates from patient L demonstrated only moderate-level resistance in spite of mutations in both *rdxA* and *frxA*. Furthermore, five isolate sets with MICs of > 256 mg/L had mutated *frxA* only, indicating that high-level resistance can occur in isolates with apparently unaltered *rdxA*. However, as the *frxA* mutations were observed in MtzS strains also, they were unlikely to contribute to resistance. Evidence presented in this chapter suggested that mutations in *rdxA*, *ahpC* and *frxA* may not be essential to development of MTZ resistance. Overall, examination of the MICs determined for all isolates in relation to mutations in these genes would also support that observation.

The results presented in this chapter suggest that the area of MTZ metabolism and resistance in *H. pylori* offers many possibilities for further investigation. Future studies could focus on the potential role of transcriptional regulation of candidate genes including those described in this thesis. Techniques such as primer extension, generation of promoter:reporter gene fusions and mRNA quantification were key in a series of recent studies demonstrating that expression of *ureA* and *ure B* genes is upregulated by a complex of $Ni^{2+}$ ions and a NikR protein orthologue that binds to motifs in the *ureA* promoter, facilitating transcription by RNA polymerase (Davies *et al.* 2002;van Vliet *et al.* 2001;van Vliet *et al.* 2002). To date, no studies have examined the promoter sequences or the transcriptional regulation of RdxA. Given the evidence to suggest that RdxA protein is not expressed in MTZ-R isolates (Latham *et al.* 2001), it would be interesting to investigate this further, possibly by an approach similar to that described above for *ureA* and *ureB*.

## 10.4 Conclusions

In the work presented in this chapter, PCR was a key technique not only in the examination of *rdxA*, *frxA* and *ahpC* in a unique strain collection but also in strain genotyping by AFLP and in rapid screening of a larger study population for specific mutations by real-time PCR. Sequence analyses demonstrated for the first time that while mutations occur in both *rdxA* and *frxA*, these may not necessarily be contributing to the MTZ-R phenotype. In contrast, no mutations were observed in *ahpC* of any matched strain pairs. There has been sufficient evidence presented previously to merit continued investigation of the potential role of these genes in MTZ metabolism and resistance development. However, a PCR-based approach has demonstrated that resistance may develop by mechanisms other than gene mutation and so development of a simple molecular test for MTZ resistance in *H. pylori*, analogous to the CLA susceptibility test described in Chapter 5, does not yet appear feasible. Continued investigation of alternative regulatory mechanisms for all three genes as well as other key enzymes at the transcription, translation and post-translational level will be essential in order to improve understanding of the MTZ resistance mechanism(s).

# Chapter 11: General discussion and conclusions

## 11.1 PCR: General considerations

Since it was first described in 1983, the PCR has revolutionised clinical bacteriology, both in diagnostic applications and as a research tool. The speed and high sensitivity of the technique along with its potential to examine organisms irrespective of their metabolic state has meant that it has been particularly useful for the study of organisms that are slow or difficult to grow by conventional means, such as *H. pylori* and other species of *Helicobacter*. The work presented in this thesis aimed to evaluate and develop both conventional and real-time PCR to provide tools for enhanced surveillance of helicobacters infecting humans and specific genotypic markers associated with virulence or antibiotic resistance of *H. pylori*.

The analyses of clinical specimens described herein raised several issues regarding the potential, and also the possible limitations, of PCR based investigation of *Helicobacter* infections. Overall, results indicated that the following factors significantly affected the success of PCR in detection and further strain characterisations.

### 11.1.1 Specimen quality

In Chapter 3 it was shown that the sensitivity of detection from gastric biopsies for different *H. pylori*-specific PCR assays was significantly reduced if the transportation of biopsies was prolonged in sub-optimal conditions (e.g. >4 °C). The adverse effects of these transport conditions were also demonstrated in subsequent PCR-based analyses of gastric biopsies, for identification of markers of antibiotic resistance (Chapter 5) and virulence (Chapters 6 and 7), where optimal results were obtained from specimens that had been stored, without delay, at low temperatures.

Consequently it was concluded that unless DNA extraction could be performed the same day as specimen collection, all clinical samples should be immediately stored (-20 °C) and low temperatures maintained during subsequent transportation and storage until further processed. These measures to ensure the availability of good quality template DNA were subsequently applied for all other specimens studied (biopsies from extra-gastric sites and stool specimens).

### 11.1.2 Method of DNA extraction

A method must be selected on the basis of the method's effectiveness at removing substances inhibitory to PCR, whilst still recovering high yields of target DNA. Other important considerations include overall processing time and labour and consumables costs. Preliminary experiments on gastric biopsies in Chapter 3 suggested that a simple DNA extraction method (homogenisation followed by digestion and boiling) met these criteria. Internal control PCR analyses in Chapter 9 subsequently demonstrated that this method was sufficient to remove PCR inhibitory substances from bladder and colonic biopsies, while the vascular bronchial biopsies were processed successfully by a commercial spin column-based kit. In contrast, faecal specimens were particularly refractory to molecular analyses as they contain high levels of competing DNA from the host and commensal flora as well as numerous substances such as polysaccharides, bile salts and bilirubin that are inhibitory to the PCR reaction. Thus, as illustrated in this study, the method of DNA extraction is critical to the success of *H. pylori* detection. Comparison of both manual and automated (MagNA Pure system) methods of DNA extraction in Chapter 8 demonstrated that overall the most successful method for both inhibitor removal and DNA recovery was manual extraction using GuSCN buffer containing milk protein

and diatoms, followed by post-extraction treatment with PVP. This is a novel modification of a previously described method (Lawson *et al.* 1997).

### 11.1.3 Primer choice for PCR-based detection

Numerous genus and species-specific PCR assays have been described previously for the detection of helicobacters, from a range of clinical and environmental specimens. However, primer binding efficiency and assay performance depends not only on the PCR conditions, but also on the individual primer properties and the level of sequence heterogeneity at the primer-binding site of the target gene. This was illustrated at several points in this thesis. In Chapter 3, sensitivity and specificity of *H. pylori* detection from gastric biopsies by a novel species-specific assay targeting *vacA* was significantly higher than the assay of Brisou *et al* (1990) amplifying *glmM* and marginally superior to the assay reported by Ho *et al* (1991) targeting 16S rDNA. Similarly, in Chapter 8, comparison of three nested PCR assays (both in conventional and real-time format) targeting *ahpC*, 16S rRNA and *vacA* genes showed that the *ahpC* assay was the most sensitive for PCR-based detection of *H. pylori* from stools. In Chapter 9, sensitivity and particularly specificity of the 16S rRNA assay by Bohr *et al* (1998) was poor compared with another genus-specific assay described by Logan *et al* (2001), while in Chapter 7, the CagMotC assay was by far the most sensitive of the three LightCycler assays developed to detect *cagA* tyrosine phosphorylation motifs (TPMs) when applied to gastric biopsies. In addition, sequencing of 16S rDNA amplicons generated from bladder biopsies in Chapter 9 demonstrated sequence mismatch at the binding site of the Hp2 primer of Ho *et al* (1991), highlighting the potential pitfalls of detection of an inherently heterogeneous organism like *H. pylori* by a single PCR assay.

An appreciation of differential assay performance is essential, therefore, when selecting appropriate PCR tests for a given application and was critical to the interpretation of results generated in the study of extra-gastric disease in Chapter 9.

### 11.1.4 Target sequence for probe hybridisation melting point analysis

Real-time PCR is significantly faster and less labour intensive than conventional PCR. The utility of the LightCycler instrument for the rapid and simple detection of sequence polymorphisms in a single reaction was demonstrated in Chapters 5, 7 and 10. However, these studies also showed that such probe hybridisation melting point analyses are most suitable for conserved genes where mismatches with probe are due to specific mutations only (e.g. 23S rDNA). Interpretation is more difficult when this approach is applied to genes or regions of genes that are inherently variable (e.g. *cagA*). This is an important consideration for many regions of the *H. pylori* genome, and novel assay design requires prior knowledge of sequence data from multiple strains. Specific sequence polymorphisms that are located in areas of high heterogeneity are unlikely to be amenable to this kind of analysis.

## 11.2 The role of PCR in the investigation of human *Helicobacter* infection

It is evident that various factors require careful consideration to ensure that PCR can provide a useful adjunct for enhanced investigation and surveillance of human *Helicobacter* infections. Nevertheless, this study demonstrated the utility of both conventional and real-time PCR for detection of viable and non-viable helicobacters from both gastric and extra-gastric sites. Additionally, PCR, particularly the real-time LightCycler format, was shown to be a powerful tool in strain characterisation.

### 11.2.1 Detection of helicobacters from gastric biopsies

In addition to the obvious advantage of rapid, sensitive and specific same-day diagnosis of infection, the power of PCR for enhanced detection of helicobacters in gastric biopsies was demonstrated in this study. Not only was it possible to detect non-viable *H. pylori* in biopsies collected for CLO test, but a novel PCR assay was developed also to detect '*H. heilmannii*'-like organisms (HHLOs) that are often non-cultivable and so have hitherto been detected in the human gastric mucosa primarily by histology. Application of this assay to gastric biopsies from dyspeptic patients in South East England demonstrated a higher rate of infection (2.3 %) than had been reported previously in most other countries, either indicating a higher geographical prevalence or highlighting inadequate sensitivity of the histology-based approach. Combination of this assay with a novel *vacA H. pylori*-specific assay produced a multiplex test (HpHh), that could detect the two principal groups of human gastropathogen with sensitivity and specificity comparable to those determined for each individual assay. At the time of writing, this PCR assay was the only one if its kind that could detect this range of helicobacters simultaneously. This allowed the first PCR-based assessment of the incidence of *H. pylori* and HHLO co-infections - the failure to identify any indicated that they are comparatively rare in adult dyspeptic patients in South East England. This novel assay will facilitate future studies to assess the significance of HHLO infection, either singly or as part of a co-infection, in relation to human gastric disease.

### 11.2.2 Further strain characterisation

Culture-based analysis of gastric biopsies offers the distinct advantage of providing *H. pylori* isolates for strain analyses, not only for surveillance of infection but also to determine strain virulence potential and antibiotic susceptibility. The ability of PCR

to allow further strain characterisation is a key benefit of this method, particularly as relatively few centres, in the UK or elsewhere, perform culture.

#### 11.2.2.1 Antibiotic resistance

Successful *H. pylori* eradication is threatened by the apparently global trend for increased prevalence of isolates that are MTZ and/or CLA resistant. Development of PCR-based methods for application to clinical specimens that are not currently undergoing culture would promote enhanced surveillance of antibiotic resistance, as well as improving individual patient management. The simplicity of the CLA resistance mechanism in *H. pylori* has facilitated development of a range of molecular susceptibility tests. The work in this thesis presents one of the first evaluations of real-time PCR-based methodologies for determination of CLA resistance directly from gastric biopsies. CLA susceptibilities were accurately determined in 95.6 % of the 62 biopsies tested, for which resistotype had been determined previously by culture-based methods. Discrepant results may occur in the case of mixed-susceptibility infections. Nevertheless, given that real-time PCR offers rapid, same-day determination of CLA resistotype in a simple, single reaction, future studies should focus on the development of sensitive assays that identify mixed infections. Additionally, following the recent report that tetracycline (TET) resistance occurs by point mutations in the 16S rRNA gene (Trieber and Taylor 2002), a LightCycler test analogous to assay LC-CLA could be developed to monitor these mutations.

In contrast, the mechanism of both MTZ action and resistance development remains poorly defined and this has hindered development of a molecular assay for rapid susceptibility testing. Nevertheless, PCR proved crucial in this study for further examination of the MTZ resistance mechanism. Two genes, *rdxA* and *frxA*, that are considered to be associated with MTZ resistance were investigated, as was *ahpC*. A

unique strategy was employed, examining not only paired pre and post-treatment clinical isolates, but also MTZ-R sub-populations of mixed resistotype infections pre-treatment. The results of PCR-based AFLP genotyping of matched isolates showed that mixed susceptibility infections pre-treatment were always phenotypic strain variants rather than mixed strain infections. Furthermore, treatment failure in this patient study group generally occurred due to persistence of the existing infection, possibly due to the selection of a pre-existing MTZ-R sub-population in many cases. Comparison of *rdxA* sequences of MTZ-S (pre-treatment) and MTZ-R (post-treatment) confirmed the findings of previous studies that mutations occurred in the MTZ-R gene (Goodwin *et al.* 1998;Kwon *et al.* 2000;Marais *et al.* 2003). Interestingly, these were not observed in the matched MTZ-R pre-treatment strains, providing the first strong evidence that the contribution of mutations in the *rdxA* gene to MTZ resistance may not be as significant as had been proposed previously. In contrast, *frxA* was identical in all populations in the majority of cases but potential inactivation of the gene by frameshift mutation, particularly at nucleotide 53, was a frequent event, even in MTZ-S strains. Development of a novel real-time PCR assay for rapid screening of a larger study population of 119 isolates from across the UK for frameshift mutation 53 confirmed that this was observed in both MTZ-R and MTZ-S isolates. The high incidence of mutationally truncated *frxA* in MTZ-S isolates suggests that inactivation of this gene alone does not cause MTZ resistance. Previous studies have indicated that *frxA* inactivation only contributes to the high-level resistance of isolates that also contain a mutated *rdxA* gene (Jeong *et al.* 2000;Jeong *et al.* 2001). That was not substantiated by the work in this thesis, where isolates were characterised that had high MICs but contained unaltered *rdxA* and mutated *frxA*. These results suggest that other enzymes are likely to contribute to MTZ resistance,

but the demonstration that *ahpC* gene sequences are identical regardless of MTZ phenotypes indicate that mutational inactivation of alkyl hydroperoxide reductase does not play a significant role. Future studies to examine regulation of RdxA, FrxA and AhpC protein expression at the transcriptional and translational level might elucidate the role of these in MTZ resistance, as could examination of other candidate enzymes such as FdxA, FdxB, PorA and PorB in this isolate set.

#### 11.2.2.2 Strain virulence

Although not well understood, it is clear that the pathogenesis of *H. pylori* infection is complex and multifactorial, relating to bacterial, host and environmental factors (Gerhard *et al.* 2002;Moran *et al.* 2002;Suerbaum and Michetti 2002). The clinical outcome of infection is variable between patients, but identification of specific virulence factors may provide useful markers for predicting disease progression, and can be used to identify patients that might benefit from eradication therapy. VacA and CagA proteins are recognised as probable virulence factors but the precise role of these in disease progression is still not clearly defined as they may differ globally according to the particular human population.

The signal and mid-region genotype of *vacA* is one feature that has been suggested to contribute to pathogenesis. To facilitate surveillance of this, a novel assay was developed that could determine *vacA* genotypes that were accurate and easy to interpret direct from gastric biopsy samples, as well as being more rapid and economical than the currently favoured uniplex methods of *vacA* genotyping. Analysis of biopsies from South East England demonstrated that type s1m1 in particular was observed frequently in isolates associated with peptic ulcer disease, a finding substantiated by studies from other countries (Arents *et al.* 2001;Kidd *et al.* 1999). Development of this convenient methodology should promote more extensive

investigation of the relationship between *vacA* genotype and disease status, although future studies should consider other bacterial and host factors to fully elucidate this complex process.

Recently the CagA protein has been shown to be secreted into epithelial cells where specific motifs such as putative TPMs A, B and C are phosphorylated by host tyrosine kinases, leading to reorganisation of the host cell cytoskeleton (Higashi *et al.* 2002;Odenbreit *et al.* 2000;Stein *et al.* 2002). While this is thought to play a role in development of gastric carcinoma (GC), the precise role of this in pathogenesis is not yet defined. In this thesis, three real-time PCR assays were developed to allow high-throughput screening of isolates for TPMs A, B or C, as a faster and more economical alternative to sequencing. Generally, the performance of each assay depended on the level of sequence variation in that region of *cagA,* but in the majority of strains there was good correlation between LightCycler assays and sequencing. Some discrepancies were observed between the LightCycler CagMotA assay and results of two PCR-RFLP assays, whereas the CagMotC assay was superior to the PCR-RFLP assay developed for TPM C. The analysis of 84 isolates of *H. pylori* from dyspeptic patients with different diseases, demonstrated that prevalence of TPM A was high in South East England isolates while TPMs B and C were comparatively rare. No clear associations between TPMs and disease status were apparent, although few isolates from patients with GC were examined. The demonstration that TPM status could be determined directly from gastric biopsies also showed that these assays could provide a useful tool for more extensive study in the UK and elsewhere. Nevertheless, this study constitutes the largest survey conducted to date. Further investigation of isolates from GC patients as well as the development of methods to screen for

additional TPMs such as the EPIYA repeats may improve understanding of the relationship between CagA phosphorylation and severity of disease.

### 11.2.3 Non-invasive detection of helicobacters

Gastric biopsies are collected by endoscopy, an invasive and costly procedure. Consequently, only a subset of the dyspeptic population (generally those > 55 years of age or those with 'alarm' symptoms suggesting malignancy) are examined. The development of non-invasive methodologies, based on analysis of stool specimens would not only reduce the risk to the individual patient but would increase access to a wider cross-section of the dyspeptic population. Furthermore, non-invasive testing provides a means for the examination of the healthy population, that at present has been studied by serology-based methods only. In this thesis, detection of *H. pylori* antigen and DNA from stool specimens was compared with culture and histology of matched gastric biopsies. At the time of writing, most studies had evaluated performance of the HpSA ELISA kit for antigen detection (Gisbert and Pajares 2001). However, the work presented in this thesis demonstrated that performance of the Amplified IDEIA HpStAR kit was superior to that of the HpSA kit, confirming the results of the few studies described to date (Koletzko *et al.* 2003;Leodolter *et al.* 2002;Makristathis *et al.* 1998;Makristathis *et al.* 2000). Additional evaluation of a simple immunochromatographic test, the ImmunoCard STAT! HpSA, demonstrated for the first time that it could reliably detect stool antigen from specimens that had generated high ODs by either ELISA, but was insufficiently sensitive for stools that had been weakly positive by ELISA. PCR-based detection of *H. pylori* was less sensitive and specific than specific antigen tests, and as discussed (section 11.1), the success of this was affected substantially by the method of DNA extraction and the individual assay applied. Nevertheless, it was shown that adaptation of the second

round of nested PCR to a real-time format did not alter the assay performance, but did reduce the overall test time. As PCR offers the clear advantage of enabling further strain characterisation, particularly in a real-time format, future studies should focus on improving sensitivity of detection from stools, by further evaluating methods of DNA extraction and PCR assay format.

### 11.2.4 Investigation of extra-gastric diseases

The potential association between helicobacters and the chronic inflammatory conditions Interstitial Cystitis (IC), Inflammatory Bowel Disease (IBD) and Chronic Persistent Cough (CPC) were examined by PCR. As discussed, unlike many preceding studies, the strategy was to apply a panel of different *Helicobacter* genus and species specific assays, to overcome false-negatives due to heterogeneity at the primer binding site and also to provide corroborative evidence of amplicon specificity, together with sequencing. This approach was particularly important as unlike the studies on gastric biopsies and stool specimens, where the availability of matched cultures and/or histological results allowed validation of novel assays or novel assay applications, there were no such "gold standard" methods available to validate assay performance from these novel extra-gastric sites. No specific DNA was amplified in biopsies from IBD or CPC patients, suggesting that helicobacters are not involved in the pathogenesis of these conditions. In contrast, specific DNA was amplified by two or more PCR assays in 18.5 % of bladder biopsies. Inter-assay discrepancy of positive results were observed for these that may have been due to differences in assay performances as discussed in section 11.1. Sequencing and BLASTn analysis indicated that amplicons had highest identity with *H. pylori* DNA. These results provide the first evidence of the presence of helicobacters in the human bladder, although no association between this and IC could be identified. The fact

that BLASTn analysis does not provide an exhaustive comparison with all bacterial and human DNA sequences means that the possibility of amplicon misidentification cannot be fully excluded. For this reason, future studies should aim to provide direct evidence of *H. pylori* at this site (e.g. by culture, microscopy, *in situ* hybridization or *in situ* PCR).

## 11.3 Concluding comments

This study has demonstrated the value of PCR-based methodologies as informative diagnostic tools for the characterisation of *Helicobacter* infections. It also has highlighted aspects of the technique that require careful evaluation and control to optimise quality of results. In the future the PCR-based tools developed during this study hopefully will facilitate new clinical and epidemiological investigations of a broader cross-section of the population than hitherto has been possible. Such studies are essential to improve our understanding of the prevalence and virulence of a genomically diverse species like *H. pylori* and of other *Helicobacter* species which are being associated with a variety of chronic human infections.

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## Appendix A: *Helicobacter* strains used to check assay specificities and for assay controls

### A.1 Strains used for evaluation of assay HHLO-16 specificity

| | |
|---|---|
| *H. pylori* | NCTC 11637[T] and NCTC 11638, strains 26695, J99, and H566 |
| *H. fennelliae* | NCTC 11612 [T] and NCTC 11613 |
| *H. cinaedi* | NCTC 11611 and NCTC 11614 |
| *H. pullorum* | NCTC 12825 and NCTC 12826 |
| *H. canis* | NCTC 12739 [T] and NCTC 12745 |
| *H. acinonychis* | NCTC 12688 and NCTC 12689 |
| *H. felis* | NCTC 12436 [T] |
| *H. hepaticus* | NCTC 12886[T] |
| *H. nemestrinae* | NCTC 12491 [T] (= *H. pylori*) |
| *H. muridarum* | NCTC 12714 [T] |
| *H. pametensis* | NCTC 12888 and NCTC 12889 |
| *H. mustellae* | NCTC 12032 |

[T] indicates the type strain of the species.

### A.2 Control strains of *H. pylori*

#### A.2.1 *vacA* genotyping assay

| | |
|---|---|
| s1m1 | NCTC 11637 |
| s2m2 | Tx30a |

#### A.2.2 LightCycler LC-CLA assay

| | |
|---|---|
| Wild type 23S rDNA | NCTC 11637 |
| Mutated 23S rDNA (A→G) | H197* |
| Mutated 23S rDNA (A→C) | H235* |

*Clinical isolates from the Helicobacter Reference Unit collection

#### A.2.3 LightCycler assays CagMotA, CagMotB and CagMotC

Cag TPM A + NCTC 11638 and 26695
Cag TPM A - NCTC 11637, ATCC43526 and J99

Cag TPM B + 26695
Cag TPM B - NCTC 11637, NCTC 11638, ATCC43526 and J99

Cag TPM C + NCTC 11637 and ATCC43526
Cag TPM C - NCTC11638, 26695 and J99

## Appendix B: Accession numbers of sequences retrieved from NCBI Entrez Nucleotide for primer and probe design.

### B.1 Complete *vacA* sequences (for design of assays HpVac and HpHh)

| | | | |
|---|---|---|---|
| U95971 | AF049647 | AF049636 | AF049625 |
| UO5676 | AF049646 | AF049633 | AF049620 |
| UO7145 | AF049645 | AF049632 | AF071097 |
| AF049652 | AF049644 | AF049631 | AF071096 |
| AF049651 | AF049643 | AF049629 | AF071095 |
| AF049650 | AF049642 | AF049628 | AF001358 |
| AF049649 | AF049641 | AF049627 | |
| AF049648 | AF049638 | AF049626 | |

### B.2 16S rRNA (For design of assay HHLO-16)

| | |
|---|---|
| '*H. heilmannii*' | AF25625, AF058777, AF058770, AF058771, Y18028, AF058773, AF058772, AF058774, AF058775, AF058776, L10079, L10080 |
| *H. felis* | M57398, M37643, U51872, U51871, U51870, AF103882, AF103881, AF 103880, AF103879 |
| *H. bizzozeronii* | Y09404, AF103883, AF302107 |
| *H. salomonis* | U89351 |
| *Candidatus* H. suis | AF127028 |
| *H. pylori* | M88157, UO1330, UO1331, UO1332, UO8906, AJ011431 |
| *Flexispira rappini* | AF034135, AF047848 |

| | | | |
|---|---|---|---|
| *H. bovis* | AF127027 | *H. bilis* | AF214508, U51873, AF047844 |
| *H. cholecystus* | U46129 | *H. canis* | L14634, UO4344, AF177475 |
| *H. hepaticus* | L39122 | *H. cinaedi* | AF207738, AF207739, M88150 |
| *H. muridarum* | M80205 | *H. mustellae* | M35048 |
| *H. pullorum* | AF047850 | *H. rodentium* | U96297 |
| *H. trogontum* | U65103 | *H. ulmiensis* | AJ007931 |

### B.3 *cagA* sequences (for design of TPM-A, TPM-B and TPM-C LightCycler assays)

| | | |
|---|---|---|
| AF202973 | AF202972 | AF247651 |
| AE000569 | AE001483 | AF249275 |
| AF282853 | AB003397 | |

### B.4 *rdxA* sequences (for design of primer )

| | | |
|---|---|---|
| AF184267 | AF184269 | AF184266 |
| AF184268 | AF012553 | AF012552 |

## Appendix C: Accession numbers of sequences retrieved from NCBI Entrez Nucleotide for alignment with sequences determined in this study

### C.1 *rdxA* sequences (to identify novel mutations)

| | | | |
|---|---|---|---|
| AF315501 | AF180418 | AF180419 | AF180420 |
| AF180421 | AF180422 | AF180423 | AF180424 |
| AF3233015 | AF323014 | AF305358 | AF305357 |
| AF323017 | AF323016 | AF184267 | AF184266 |
| AF184268 | AF012553 | AF012552 | AF184269 |
| AJ305346 | AJ305347 | AJ305348 | AJ305349 |
| AY063489 | AY063488 | AJ305356 | AF180395 |
| AF323003 | AF323002 | AF180396 | AF180397 |
| AF180398 | AF180399 | AF180400 | AF323005 |
| AF323004 | AJ305350 | AJ305351 | AF323019 |
| AF323018 | AF323007 | AF323006 | AF180401 |
| AJ305355 | AF180402 | AF180403 | AF180404 |
| AF3230009 | AF3230008 | AF180405 | AF180406 |
| AF180407 | AF180408 | AF180409 | AF180410 |
| AF180411 | AF180412 | AJ305352 | AJ305353 |
| AF180413 | AF180414 | AF180415 | AF315502 |
| AF180416 | AF316109 | AF323011 | AF323010 |
| AF323010 | AF323012 | AF180417 | |

### C2 *frxA* sequences (to identify novel mutations)

| | | | |
|---|---|---|---|
| AF322960 | AF322959 | AF322964 | AF322963 |
| AF183174 | AF183175 | AF183176 | AF322966 |
| AF322965 | AF322976 | AF322975 | AF322974 |
| AF322973 | AF183392 | AF322972 | AF322971 |
| AF322962 | AF322961 | AF225923 | AF322968 |
| AF322967 | AF322970 | AF322969 | |



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